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Stimulation of substance P release from sensory nerves by incretin hormones

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Abstract

Stimulation of substance P release from sensory nerves by incretin hormones

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The communication between the gut and the brain is very complex and is regulated by various systems, such as the endocrine, immune, autonomic, and the enteric nervous system. Food intake has been known and thought to play a major role in this communication. Previously the laboratory of my graduate work showed that guanine nucleotide-binding protein couple receptor (GPCR) GPR93 (also known as lysophosphatidic acid 5 (LPA5) or GPR92) is located at the apical side of the gut lumen and senses nutrients in the lumen of the gut. This receptor was the first to be discovered to be localized on sensory nerves and yet respond to components in mesenteric lymph fluid (MLF) induced in response to the diet. The lymphatic fluid contains cells and molecules from the interstitial fluid (IF) that surrounds specific tissues. My study was to examine if the afferent sensory nerves that innervate the mesenteric lymphatic vasculature could be activated by diet induced molecules of the MLF to form a system of nutrient sensing of the interstitium. I observed that MLF stimulated primary cultured mouse dorsal root ganglia (DRG) sensory neurons. I discovered that Glucagon-like peptide 1 (GLP-1) or Glucose-dependent insulinotropic polypeptide (GIP) as model molecules of the MLF stimulated those neurons resulting in the release of Substance P (SP). GLP-1 and GIP are incretin hormones, which induce blood glucose levels to go down after a meal. The results of this study showed a new mode of action for GLP1 and GIP incretin hormones that is extra-pancreatic and how the peripheral tissues and the central nervous system could be stimulated by molecules of the interstitium/MLF via afferent sensory nerves.

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GPCR: guanine nucleotide-binding protein couple receptor

LPA5: lysophosphatidic acid 5

MLF: mesenteric lymph fluid

IF: interstitial fluid

DRG: dorsal root ganglia

GLP-1: Glucagon-like peptide 1

GIP: Glucose-dependent insulinotropic polypeptide

SP: Substance P

GI: gastrointestinal

PYY: peptide tyrosine-tyrosine

NT: neurotensin

CCK: cholecystokinin

EECs: enteroendocrine cells

PC1: prohormone convertase 1

PC2: prohormone convertase 2

MPGF: major proglucagon fragment

DPP-4: dipeptidyl peptidase IV

GLP-1R: GLP-1 receptor

GIPR: GIP receptor

CNS: central nervous system

PNS: peripheral nervous system

CGRP: Calcitonin gene-related peptide

TRP: transient receptor potential

TRPC1-7: canonical

TRPV1- 6: vanilloid

TRPM1-8: melastatin

TRPA1: ankyrin

TRPP1-3: poly-cystin

TRPML1-3: mucolipin

TRPV1: transient receptor potential vanilloid 1

TRPA1: transient receptor potential ankyrin

AITC: isothiocyanate

Background

Various cultures have used different foods for their assumed and/or known effects. I was brought up in a culture that categorizes certain foods as “cold” or “warm,” on how they affect the body. In 400 B.C., the “Father of Medicine”, Hippocrates, pronounced, “Let thy food be thy medicine and thy medicine be thy food.” This Greek physician pointed to the significant impact of food on a person’s body and mind to prevent illnesses, to maintain health and wellbeing. The Bedouin, the people of the desert, of the ancient stated, “you are what you eat.” The ancients already recognized that food was not only for their survival but ingested food also communicated with the mind. The link between food and health, or how ingested food communicates with the body and the brain, has been investigated from a very ancient time, and still there is much left yet to be discovered.

Nutrient Sensing

Digestion and nutrient sensing begins at the mouth. Even with the thought or sight of food saliva is secreted and with food in the mouth and chewing, the salivary glands are further stimulated to release more and more saliva, depending on the size of the bit and the strength of the chewing. Saliva makes compounds accessible for taste by breaking down food (1). After the ingestion of food various metabolic responses take place in the gastrointestinal (GI) tract, including the secretion of hormones. The GI tract is the largest endocrine organ of the body. Specific mucosal responses are initiated by the ingested macronutrient and micronutrient components. Micronutrients are substances in food that the body needs in trace amounts to function properly, such as vitamins and minerals. Unlike Macronutrients, they yield no energy (2). Macronutrients are the components of food, such as carbohydrates, fats, and proteins, that provide our body with calories or energy (3) (4). Multiple hypotheses exist on the mechanism of macronutrient sensing. Our lab previously reported that dietary macronutrients in the lumen of the intestine could influence what takes place in the intestinal mucosa via receptors. Gastrointestinal bioactive peptides, such as cholecystokinin (CCK) and peptide tyrosine tyrosine (PYY), are shown to be secreted by the stimulation of macronutrient and subsequently activate their receptors (5). Layer and colleagues first reported in 1995 on Glucagon-like peptide 1 (GLP-1) being released in the ileum in response to carbohydrate and lipid perfusions. GLP-1 was previously discovered as a hormone that regulated gastric secretion(6) (7). In 1998, Dumoulin and colleagues published on the release of peptide hormones, including PYY, GLP-1, and neurotensin (NT), in response to specific nutrients being infused in the rat ileum (8). Even before Dumoulin, the release of PYY was investigated by Aponte, who concluded that the release of the peptide hormone is not dependent on the length of the fatty acid chain and that the duodenum is not the major site of release of this peptide (9).

PYY and other hormones of the gut play a role in what is termed the “ileal break”, which is the most studied and earliest discoveries on the effects macronutrients, in this case dietary fat, have on how the function of the intestine is regulated. The “ileal break” is a negative feedback mechanism that controls the rate at which ingested food moves through the gut, it decreases the motility of the GI tract in response to nutrients that are not absorbed and decreases food intake and results in a sensation of satiety. Very

effective inhibitors of gut motility and digestive enzyme secretion are long chain fatty acids. This is, to some extent, because of their stimulation of regulatory enteroneurohormone secretion, like CCK and PYY. That occurs when the content of the GI lumen comes in contact with the L and N cell of the gut mucosa resulting in secretion of those hormones. This is how the ileal brake is thought to regulate digestion and absorption of food (10) (11).

The mechanism of macronutrient sensing was first reported by the discovery of nutrient-sensing G protein coupled receptors (GPCRs), which were shown to be localized at the brush border enteroendocrine cell plasma membranes. And conveniently they were shown to face into the gut lumen allowing them to be stimulated by post-prandial luminal contents, such as peptide hormones (11) (12) (13) (14) (15).

Nutrient sensing GPCR

In the last decade, various seven transmembrane receptors have been identified and characterized that sense organic nutrients. The ligands for such receptors include amino acids (taste1 receptor T1R1/T1R3), proteolytic degradation products (GPR93, also termed GPR92), carbohydrates (T1R2/T1R3 receptor), and free fatty acids (FFA1, FFA2, FFA3, GPR84, and GPR120). They are expressed in a variety of tissues, such as taste tissue, the gastrointestinal tract, endocrine glands, adipose tissue, and the kidney. These receptors have the ability to act as sensors of food intake and function in regulating the release of incretins and other hormones from the gut, insulin and glucagon from the pancreas, and leptin from adipose tissue (16).

GPCR

GPCRs account for a large and diverse family of cell surface proteins that are present in the GI tract, taste tissues, endocrine glands, adipose tissues, kidney and others. There are more than 800 GPCRs found in humans, which fall into the following five classes: rhodopsin (Class A), secretin (Class B), glutamate (Class C), adhesion (Class D), and frizzled/smoothened (Class E) (17) (18,Devi, 2005 #90). GPCRs are comprised of seven transmembrane domains, in addition to an external amino terminal fragment, which interacts with a ligand, and an internal carboxy terminal end, which interacts with the intracellular G protein. They are significant molecules in our sense of smell, taste, touch, and vision (10).

They sense signals in their extracellular environment and transmit these to their appropriate intracellular heterotrimeric G proteins. When these G proteins are in their GDP containing heterotrimeric state, they are inactive. They become active when the heterotrimeric protein, Gαβγ, separates into the active Gα and Gβγ dimer subunits as a result of nucleotide exchange, and GDP is converted to GTP by the endogenous GTPase activity of the G proteins. There are different groups of Gα, such as $G\alpha_{q,}$ that increases intracellular calcium concentrations and $G\alpha_{s}$ that enhances cAMP generation. There are various different cascades that are induced by the downstream effects of the ligand interaction, and the activation of the Gα protein and the Gβ/Gγ dimer. Three most common cascades that could be activated by this are the adenylate cyclase/cAMP, MAPK kinase, and phospholipase C mediated pathways (19) (20) (18) (21) (22) (23) (24).

Peptide hormones and gut peptides

A major class of hormones are classified as peptide hormones, which are ligands to all Class B or the rhodopsin class GPCRs, such as glucagon, parathyroid hormones, and calcitonin. They serve as leading targets for drug therapies, such as for endocrine and neuronal disorders (17). Peptide hormones play significant role in various in vivo processes, such as being important signaling molecules. In vivo, enzymes break down peptides quickly, and thus they are rapidly excreted from the body.
They are made by specialized endocrine glands, as part of a more complex large preprohormone, which is then proteolytically cleaved at its N-terminal signal sequence and results in a prohormone. This prohormone matures, as it is transported from the rER to the Golgi apparatus, and finally enclosed in secretory granules to be released as the bioactive hormone. As they are released into the circulation, the peptide hormones find their receptors located on the plasma membrane of the target cells and interact with them to initiate an intercellular signaling pathway that will regulate some specific physiological process. A few of their target regulations may be energy metabolism, growth, stress, or reproduction. Overexpression of peptide hormones are related to tumors and used as clinical diagnosis for monitoring their growth and disease process (25) (26).

One major class of peptide hormones are the gut peptides. The physiological importance of the gut peptides has been much broadened over the last decade, then just the gastrointestinal digestion and nutrients absorption. Specialized cell in the gastrointestinal tract called enteroendocrine cells (EECs) make up around 1% of the total gut epithelium cell population. They release more than 20 signaling molecules that have endocrine and metabolic functions. These molecules are part of the gut-brain axis and are able to communicate with the brain (27) (28). Many gut hormones have been discovered and identified as being part of the gastro-entero-pancreatic system, but the first being the discovery of “secretin” by Bayliss and Starling. These gut hormones play a role in many metabolic disorders, such as obesity, type 2 diabetes, and others. Special interest has been put on the two incretin hormones, GLP-1 and Glucose-dependent insulinotropic polypeptide (GIP), which are significant in a number of physiologic process, specially glucose homeostasis (29) (30).

GLP-1

GLP-1 is an incretin hormone made in the small intestinal epithelial L-cells from posttranslational processing of a gene product called proglucagon. It was first discovered in humans as an incretin hormone in 1987. It is a 30 amino acid long peptide hormone that is secreted from these cells in response to intake of food. GLP-1 follows the evolving idea that the synthesis of small peptide hormones take place from larger prohormones and that the final form of the hormone that has biological activity is formed by posttranslational cleavages by enzymes from the prohormones. Earlier examples of such hormones include insulin and parathyroid hormones. Furthermore, GLP-1 also shows liberation that is tissue specific. Table. 1, modified from Daniel

Drucker, depicts the location of the peptides and its tissue specific liberation. However, unlike the typical bioactive peptide that follows the regular liberation process from a prohormone, being cleaved at sites having two basic amino acids and thus beginning

with histidine and having a glycine at the end, GLP-1 has a “second single basic amino acid followed by histidine residing 6 amino acids carboxyl-proximal to the histidine.” This makes GLP-1 a 31 amino acid long peptide, rather than 37, called GLP-1 (7-37). Moreover, a sequence RGRR, which suggests for a prohormone “convertase-directed cleave” site, followed by “an amidation of the penultimate arginine” via a peptidylglycine-alpha-amidation monooxygenase takes place, which produces two peptides of 36 and 30 amino acids long, namely GLP-1(1-36)$_{amide}$ and GLP-1(7-36)$_{amide}$ (31).

1	GRPP	
3	IP-1	
2	Glucagon	Pancreas (PC2)
4+5+6	MPGF	
4	GLP-1 [GLP-1(1-37), GLP-1 (7-37), GLP-1 (7-36)]	
5	IP-2	
6	GLP-2	
2+3	Oxyntomodulin	Intestine (PC1)
1+2+3	Glicentin	

Table 1. Human proglucagon structure and processing in human, depicting tissue specific liberation of each peptide derived from proglucagon in the pancreas or the intestine. PC1: prohormone convertase 1; PC2: prohormone convertase 2; MPGF: major proglucagon fragment. Modified from: (31).

The enzyme called dipeptidyl peptidase IV (DPP-4), inactivates GLP-1 very rapidly, not even allowing the hormone to leave the gut. Thus, even though GLP-1 has important functions outside the gut, it is already inactivated before it has a chance to leave the gut. This makes GLP-1 a possible neurotransmitter, where its actions are transmitted through sensory neurons in the intestine and elsewhere in the body where the GLP-1 receptor (GLP-1R) is expressed. Thus GLP-1 can be observed as a peptide hormone and a neuropeptide, having functions both as an endocrine hormone and also having neural actions (29) (30) (32).

The most well-known action of GLP-1 is its action as an incretin, which is the stimulation of insulin secretion in response to a meal and to inhibit the secretion of glucagon, therefore decreasing the glucose level found in the blood after a meal. GLP-1 is also known function as an enterogastrone by inhibiting gastrointestinal motility and section. Furthermore, GLP-1 plays a physiological role in regulating appetite and food intake. Because of the above-mentioned actions of GLP-1, GLP-1 and GLP-1R agonists are used to treat type 2 diabetes. Obesity may result when there is not enough GLP-1

secreted from the L-cells, or when the enzyme, dipeptidyl peptidase IV, is overactive. On the other hand, postprandial reactive hypoglycemia may result if there is too much GLP-1 around or the activity of DPP-4 is inhibited (29) (30) (32).

GIP

Glucose-dependent insulinotropic polypeptide (GIP) is another incretin hormone that is also degraded by of dipeptidyl peptidase IV. It was first discovered in 1973 by Brown and collages and termed gastric inhibitory polypeptide, because they observed it as a polypeptide inhibitor of gastric acid secretion in dogs that received Heidenhain's pouches. These pouches are small sacs that is closed off from the main cavity but with an opening in the abdominal wall and also vagally denervated. They are used for obtaining gastric juice (33). GIP is released from the K cells of the upper part of the small intestine. It is 42 amino acids long. Although it was first known for its inhibition of gastric acid, it was later discovered that GIP also stimulates the release of insulin, by directly acting on the islets of the pancreas in healthy individuals when administrated orally. Furthermore, it was found that in gastrectomized patients, endogenous GIP resulted in a glucose dependent release of insulin, which made it the first incretin hormone and thus it was renamed as glucose-dependent insulinotropic polypeptide. Like GLP-1, GIP also need to go through proteolytic processing of preproGIP to make the secreted GIP (32) (34) (35) (36) (37) (38). These incretin hormones also have pancreatic and exopancreatic functions that are well summarized in Table. 2, modified from (39).

	GLP	**GIP**
Brain	↑ Memory	↑ Memory
		↓ Food intake
Pancreas	↑ Insulin	↑ Insulin
	↑ Glucagon	↓ Glucagon
	↓ Beta-cell apoptosis	↓ Beta-cell apoptosis
	↑ Beta-cell proliferation	↑ Beta-cell proliferation
GI tract	↓ Gastric acid secretion	↓ Gastric emptying
Heart	↑ Cardioprotection	
	↑ Cardiac output	
Bone		↑ Bone formation
Adipose tissue		↑ Fat accumulation

Table 2. Illustration of the pancreatic and exopancreatic function of GIP and GLP-1. Modified from: (39).

Previously, it has been difficult to study GLP-1 and GIP in the circulation, because of their low levels present in the blood and also the low sensitivity of the available

immunoassays. However, in 2011, with the development of new methods, such as the lymph fistula rat model and the lymph duct cannulation techniques used in mice, these limitations were overcome and furthermore enabled scientist to follow and monitor the secretion of incretin continuously from the gut in response to food (40) (41).
GLP-1 levels in the mice gut lymphatics was evaluated after glucose ingestion versus fat ingestion in vivo, and also with or without DPP-4 inhibition. Lymph was collected every 30 minutes from the mesenteric lymphatic duct that was cannulated and compared to peptide plasma levels. Treatments were administered by gastric gavage. It was shown that basal intact levels of GLP-1 was more than 5 times higher in lymph compared to plasma (0.37 pmol/l versus 0.07 pmol/l), and basal DPP-4 activity was about 5 times lower in lymph compared to plasma (4.7+/-0.3 pmol/min/mul versus 22.3+/-0.9 pmol/min/mul). Furthermore, the flow of lymph also doubled comparing before and after glucose and fat adminstration. Calorie dependency was increased by lymph GLP-1 levels after glucose and fat ingestion, however with different patterns. Glucose caused a transient increase and fat, a sustained increase that was mainted above baseline even after 210 min. Overall, it was illustrated that there is significantly less GLP-1 in plasma compared to lymph and also there is significantly less DPP-4 activity in the lymph compared to plasma, corrlating with the increased GLP-1 concentration (42).

GLP and GIP's actions are mediated by their specific receptors, GLP-1R and GIP receptor (GIPR). These receptors are expressed in the central nervous system (CNS) as well as the peripheral nervous system (PNS), including the eyes, kidney, heart, liver, fat and several other organs (40). We are interested in the receptors that are expressed in the sensory nerves that innervate the lymphatic lacteals of the gut. Such nerves are part of the spinal nerves.

Gut-Brain Axis

The gut-brain axis has been well established through the vagus nerve, which connects the gastrointestinal tract and the brain and through afferent nerve fibers sending information about the inner organs to the brain. In addition to such communication, we provide evidence that the lymphatic lacteals of the intestine are innervated by visceral sensory nerves that are part of the spinal afferent nerves, separate from those of the vagus nerve (41). In such a system, the interstitial fluid acts as a vehicle for the molecules deposited there from the surrounding tissues to exert biological activity through these spinal afferent nerves to stimulate a specific systemic physiological response. The interstitium is made from various layer, which include the interstitial fluid, the extracellular matrix that is present on the outside of blood and lymphatic vessels, and connective tissues. Molecules from the blood capillaries, including the interstitial fluid, secretions from cells, and other factors from the surrounding cellular environment, such as metabolites, antigens and cytokines, come together into the lymphatics forming the lymphatic fluid. Some factors that determine the formation of the lymphatic fluid include transcapillary exchange that is dependent on the hydrostatic pressure of the vessel, the concentration of blood protein found within the capillaries, and the interstitium. One of the features that makes mesenteric lymphatic fluid special is the fact that it can contain molecules that come from the lumen of the intestine and are

exogenous in origin, in addition to gut peptides and other molecules secreted from the enteroendocrine cells of the intestine (43).

Sensory Nerves

The spinal nerves directly branch from the spinal cord and the CNS. They are classified as a part of the PNS. They are mixed nerves that come in pairs and have both sensory and motor fibers, and are able to transmit sensory, motor, and autonomic impulses going into and out of the spinal cord, to and from the rest of the body. Thus, they function as intermediary fibers between the CNS and the periphery. There are a total of 31 spinal nerves that are grouped based on their spinal region. There are eight cervical nerve pairs located in the cervical region, names C1-C8, twelve thoracic nerves pairs located in the thoracic region, as the name suggests, and are called T1-T12, five lumber nerve pairs (L1-L5), and one single coccygeal nerve pair (44).

The dorsal root, which synapses at the dorsal horn of the spinal cord, and the ventral root, which synapses at the ventral horn, both emerge forming the spinal nerve, from the posterior and anterior side, respectively. The dorsal root, composed of afferent sensory axons, sends visceral and somatic sensory information from the peripheral receptors back to the CNS, forming the afferent sensory root of the spinal nerve. It travels to the dorsal root ganglia (DRG), which are oval enlargement found just outside the spinal cord and house the pseudo-unipolar cell bodies of the nerve fibers. Specific spinal nerves are mapped to the human body in an organized way, dividing the body into regions, called dermatomes, which are areas of cutaneous sensory innervation by specific spinal nerves. To transmit information to the spinal nerve, the DRG will synapse on an interneuron which is found in the cord's gray matter as a part of the motor reflex arc. To transmit sensory information to the thalamus, dorsal root fibers ascent through multiple pathways in the spinal cord (45) (44).

Primary sensory neurons receive information from the external environment and then transmit it to the CNS. The activation of these neurons causes the release of a variety of neurotransmitters, such as Substance P (SP) and Calcitonin gene-related peptide (CGRP) from peripheral endings (46). CGRP and SP are two neuropeptides that are significant in the process of hyperalgesia and pain. They also play a role in migraine attacks, specially CGRP as a potent vasodilator, which is secreted during migraine attacks from sensory nerve endings (47) (48). In the trigeminovascular system, CGRP is more responsible for the regulation and transmission of pain, as opposed to SP that enhances vascular permeability (48) (49) (50) (47).

TRPV1 and TRPA

The TRPV1 and TRPA receptors are part of the transient receptor potential (TRP) family, that is made up from a diverse group of cation channels, which are responsible for the regulation of different intracellular signaling pathways. Based on their homology, the 28 mammalian TRPs are placed into 6 subfamilies, namely: canonical (TRPC1-7), vanilloid (TRPV1- 6), melastatin (TRPM1-8), ankyrin (TRPA1), poly-cystin (TRPP1-3) and mucolipin (TRPML1-3) (51). The most studied TRP channel is the transient receptor potential vanilloid 1 (TRPV1) channel, which plays an important role in nociception and sensory transmission and releases SP upon stimulation. It functions as a nonselective and selective gate for cations, where it has 10-fold higher preference for

calcium when it is activated, rather than functioning nonselectively. TRPV1 was first discovered in dorsal root ganglia and shown to be the receptor for capsaicin. However, now the receptor is illustrated to have several significant other functions, such as being activated by noxious heat over 42°, pH, lipoxygenase products, and voltage; and broad distribution within the CNS. TRPV1 can interact with other receptor pathways and it can be activated by a variety of potentially noxious stimuli. This makes the receptor a good candidate for a stress response protein, regulating the function of the CNS in response to stress. TRPV1 plays a major role in CNS functions by modulating glial and neuronal activity. It has been shown that the receptor is involved in glial reactivity, cytokine release and synaptic transmission and plasticity (51) (52).

When this calcium permeable cation channel, TRPV1, is activated, a large inward current is induced, which increases intercellular calcium concentration, caused by the influx of calcium from the intracellular compartment of primary sensory neurons. This depolarization caused by the TRPV1 was illustrated to be transmitted to the CNS as nociceptive information through primary sensory neurons (53) (54) (55). Bradykinin receptors, purinergic receptors, and glutamate receptors, are other receptors that regulate current responses that are mediated by TRPV1 (56) (57) (58) (59) (55).

The transient receptor potential ankyrin 1 (TRPA1) channel is co-expressed in some TRPV1 expressing DRG neurons. It is expressed in skin sensory neurons, epithelial cells of the intestine, lungs, urinary bladder and other places. This cation channel senses thermal and mechanical stimuli and is stimulated by allyl isothiocyanates, which are found in mustard, wasabi, and horseradish, garlic, onion, tear gas, cinnamon, etc. Ligands for TRPA1 are used to treat inflammation-associated pain (60).

Both TRPV1 and TRPA1 transmit signals to secondary neurons in the dorsal horn of the spinal cord and the brain by acting on neurons as primary afferent nociceptors. When capsaicin binds to TRPV1 or allyl isothiocyanate (AITC) binds to TRAPA1, it triggers a calcium-dependent mechanism and can cause the release their stored neuropeptides from the stimulated peripheral nerve terminals. This has been termed a “sensory-effector” function of these receptors. Calcitonin gene-related peptide and substance P are the most studied sensory neuropeptides that can be released by such activation of nerves and function as stimulators of inflammation (61). Taking this into consideration, we can specualte that molecules found in the IF/MLF could stimulate such peptidergic sensory neurons that have receptors for these molecules contained in the MLF and located in the lymphatic lacteals to secrete peptides, such as SP, when stimulated. This would provide additional evidence that molecules from the IF/MLF stimulate sensory nerves innervating the lymphtic vasculature and releasing neuropeptides as a result, thus signaling to the brain as part of a chemosensory system (62).

LPA5

LPA5 expressing cells illustrated mobilization on intercellular calcium when treated with MLF in a time- and dose- dependent fasion. It served as a model for GPCRs that are nutrient-responsive and localized on sensory nerves (62). LPA5 modulates the proliferation and differentiation of mucosal cells, as well as the secretion of hormones from intestinal cells. It was also shown by our lab that the LPA5 receptor responded to

dietary products in the lumen, namely dietary protein hydrolysate (peptone). From the literature, it is known that the secretion and transcription of cholecystokinin (CCK) is escalated by protein hydrolysate. This lead to the idea and further discovery that LPA5 plays a role in the stimulation of CCK expression and secretion via its protein hydrolysate ligand (63) (64). A calcium-dependent release and synthesis of CCK was observed with the activation of LPA5 by peptone. However, no CCK was synthesized for release when the receptor was activated by LPA, another ligand for LPA5. The presence of peptone seems to be what regulates the secretion of CCK from the enteroendocrine cells (EECs) of the gut and this release is not dependent of whether LPA is present or not (63) (64) (65). The expression of LPA5 is very broad. It is expressed in the mucosal cells, specifically the enteroendocrine cells of the duodenal-jejunal mucosa (64), neuronal tissue, including DRG (66) and the dorsal horns of the spinal cord (67).

Rationale and Objectives

Our lab previously discovered a nutrient responsive GPCR, LPA5, that is co-expressed in primary cultured DRG neurons expressing CGRP and TRPV1. The characterization of this receptor was the first to illustrate that sensory nerves express receptors that respond to changes in MLF induced by the diet, and that these peptidergic nerves innervate lymphatic lacteals of the gut, thus coming in direct contact with MLF (62-64).

The objective of my study was to determine if sensory nerves, similar to the nerves innervating the lymphatic lacteals of the gut, could be activated by MLF containing molecules derived from the interstitial fluid, as part of a chemosensory system of the interstitium. In this system, the molecules of the MLF is reflective of the IF and contains endogenous as well as exogenous molecules, such as those that originate from the lumen of the intestine. Such molecules could exert their biological activity and transmit signals through peripheral sensory nerves, to produce the appropriate physiological response. Furthermore, such activity is potentially localized, as the MLF/IF contents depends on its surrounding tissue/organ. Specifically, my aims are to examine the activation of sensory nerves by GLP-1 and GIP as models, because their concentration in the MLF is induced by feeding, and to investigate if sensory nerves stimulated with these peptide hormones would result in the release of Substance P.

Experimental procedures

Compounds. Exendin-4 (Ex-4), a potent agonist of the glucagon-like peptide 1 (GLP-1R), was purchased from AnaSpec. Gastric inhibitory polypeptide (GIP) was purchased from Phoenix Pharmaceuticals, Inc. All other chemicals were purchased from Sigma Aldrich.

Animals. WT mice, C57BL/6 (6–10 wk) from Jackson Laboratory, of equal number of males and females, were used as controls for all experiments. Procedures were carried out according to the guidelines of the National Institutes of Health Animal Research and

were approved by Institutional Animal Care and Use Committees of the University of California at Berkeley and by the University of Cincinnati Institutional Animal Care and Use Committee. The generation and background of GLP-1R KO and GIPR KO mice on a C57BL/6 background used in this study were kindly provided by Dr. Daniel Drucker as have been previously described (68). TRPA1 KO and TRPV1 KO were gifts from Dr. David Julius as described (53) (69). TRPA1/V1double-mutant mice were generated as described (70). Briefly, TRPV1–/– and TRPA1–/– animals were crossed resulting in TRPV1+/– TRPA1+/– progeny, which were then crossed to yield wild-type and double-knockout siblings for analyses.

Plasmid constructs. The GLP-1R expression vector was constructed by subcloning PCR amplified human GLP-1R open reading frame into pcDNA 3.1 (Addgene). PCR amplification was carried out using a cDNA clone from GE Healthcare (CloneId: 8327594, Accession: BC112126) as the template (forward primer, 5' – GGCCGGCCGCCCGCCATGGCCGGC-3'; reverse primer, 5' – GGAAGATCTTCCCCAGGGTCGGCTGCAGGAGGC-3'). The NK1R expression vector was constructed by subcloning PCR amplified human NK1R open reading frame into pcDNA 3.1 (Addgene). A cDNA clone from GE Healthcare (CloneID: 30915310, Accession: BC074912) was used as a template for the PCR amplification (forward primer, 5'-CGCCAAGCTTCACCATGGATAACGTCCTCCC-3';
reverse primer, 5'-CAAAGGCCGCGGGGCCAAGGAGAGCACATTGG-3'). The constructs were verified by DNA sequencing (DNA Sequencing Facility, University of California, Berkeley).

Immunocytochemistry. Gastrointestinal tissues were from adult female (n=3) and male (n=3) C57BL/6 mice. Tissues were immersion-fixed for 1–2 days at 4°C. For cryostat sections, tissues were incubated in 20– 25% sucrose in PBS for 24 hr at 4°C, embedded in OCT compound (Miles), and sectioned at 10μm. Sections were processed after mounting on slides. Prior to immunostaining, tissue sections were incubated in an aqueous solution of 0.3 % Sudan black in 70% ethanol for 5min, rinsed and processed as previously described (62). Tissues were incubated with one of the following primary antibodies: GLP-1R, mouse 1:200 (71) Developmental Studies Hybridoma Bank, Iowa: substance P, rat 1:400 Cuello et al.(72); TRPV1, guinea pig 1:1000 Julius et al. (73); podoplanin, Syrian hamster 1:400 Farr et al. (74) Developmental Studies Hybridoma Bank, Iowa; LYVE-1 rabbit 1:200 (Abcam). Tissues were then washed and incubated with secondary antibodies conjugated to rabbit or goat anti rabbit, rat, mouse, or guinea pig IgG conjugated to FITC, Rhodamine Red X, Texas Red, or horseradish peroxidase (Jackson ImmunoResearch) or conjugated to Alexa-488 or Alexa-568 (Molecular Probes; 1:200 –1:1,500 dilution, at room temperature for 2 hr). For simultaneous detection of two antigens, specimens were incubated with primary antibodies against both antigens and then secondary antibodies labeled with contrasting fluorophores. Sections were then washed and mounted in ProLong Diamond Antifade Mountants (ThermoFisher) and then coverslipped. Non-specificity was assessed by analysis of staining by omission of primary antisera.

Confocal microscopy. Specimens were observed using a Zeiss 710 laser scanning confocal microscope, or a Leica TCS-SP confocal microscope. The following objectives were used: Zeiss Fluar 20 (NA 1.0), Plan Apo 40 (NA 1.4), 100 (NA 1.3); Leica 10 (NA 0.4), 20 (NA 0.7), 100 (NA 1.4). Images were collected at a zoom of 1–2 and typically 10–20 optical sections were taken at intervals of 0.5–1.0 μm as previously described (75). Images were processed to adjust contrast and brightness using Adobe PhotoShop CS6 (Adobe Systems, Mountain View, CA) and digitally colored to represent the appropriate fluorophores (75). Images of stained and control slides were collected and processed identically.

Structured illumination microscopy (SIM). Imaging was performed in the Biological Imaging Facility at UCB using a Carl Zeiss Elyra PS.1 microscope with a 63x/1.4 and 100x 1.47 objective lenses. Excitation wavelengths were 405, 488, 561, 642. Emission wavelengths were: 420-480; 495-550; 570-620; LP655. SIM processing and Channel alignment was done using ZEN Black, with 3D rendering, colocalization, and isosurface modeling using Imaris v9 (Oxford Instruments). Processing and filtering settings were kept constant and image intensity was preserved with the raw image scale option in Zen. Colocalization analysis was performed using Huygens Pro 18.10 (Scientific Volume Imaging B.V., The Netherlands) to generate a co-localization map using the Costes method of threshold estimation with Manders coefficients of X=0.833 and Y=0.465 Images presented are representative of a minimum of five fields viewed per replicate with at least two technical replicates and the experiment was conducted in at least three biological replicates.

Mesenteric lymph fluid (MLF) extraction. MLF was extracted as previously described (62). Briefly, MLF was obtained from a cannula placed in the superior mesenteric lymphatic duct from isoflurane anesthetized and overnight fasted rats. They were presented with 3 ml-bolus of either dextrin (1.1g in 3 ml 0.9 % NaCl representing fasted lymph) or lipid rich Ensure™ (representing fed lymph, Abbott Laboratories). Ensure™ is a mixed meal consisting of fat, carbohydrate and protein. Sixty min prior to the Ensure™ infusion, MLF was collected for fasted value. MLF was taken at various time points after Ensure™ was infused into the duodenum and placed on ice. MLF collected at 120 min after the nutrient infusion (at 1:50 dilution) induced the highest increase in $[Ca^{2+}]_i$ mobilization in DRG neurons. Therefore, we used this timing of collection to represent fed MLF.

Cell culture condition and transfection. The non-tumorigenic rat mucosal epithelial cells (hBRIE 380i cells (76)) used for this study were as previously described (64). They were well-characterized subclones expressing enterocyte phenotypes. Experiments were performed using hBRIE 380i cells of passage 11-16. Cells were maintained in Iscove's modified Dulbecco's medium (IMDM; gibco) with 10% bovine calf serum (BCS; Hyclone), 100 U/ml penicillin, and 100 g/ml streptomycin (Invitrogen) as additional supplements, at 37 °C in 5% CO_2-95% air. For experiments, cells were plated on circular optical borosilicate glass coverslips (18 mm, 0.13 to 0.17 mm; FisherScientific), coated with poly-L-Lysine (Sigma-Aldrich) and placed in 12 well plate at ~80% confluency on the day of use. For transient transfection, cells were trypsinized,

resuspended in IMDM, and incubated with the plasmid DNA (3 μg plasmid DNA/1 million cells) in a volume between 0.2 and 0.7 ml at RT for 5 min. Transfection was carried out by electroporation using Gene Pulser (Bio-Rad), in 0.4-mm cuvette at 0.25 kV and 960 F. Immediately after electroporation 1 mL of IMDM-10% BCS was added to the cuvette. Cells were then plated on circular coverslips to reach ~80% confluency in 16 hr.

Neuronal cell culture and transfection. DRG were dissected and dispersed as previously described (77) with some modifications. DRG were removed from T1-T13, L1-L6, and S1-S4 segments of both sides of the spine from 4-8 weeks old mice. They were dissociated with 0.125% collagenase P (Boehringer) in CMF Hank's solution (gibco) at 37 °C for 40-60 min, pelleted (5 min at 750 x g) and resuspended in 0.25% trypsin (Invitrogen) at 37°C for 2 min. DRG were triturated gently with a fire-polished Pasteur pipette in culture medium (DMEM, gibco) with 5% Equine Serum (Hyclone), 5% FBS (Hyclone), 0.1 mg/ml penicillin– streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), then centrifuged at 750 x g for 5 min using Eppendorf 5702 centrifuge. Cells were resuspended in culture medium and plated onto coverslips coated with poly-L-lysin (PLL, Sigma-Aldrich). Cell cultures were maintained in a 5% (v/v) CO_2 incubator at 37 °C. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen), following the provider's protocols for neuronal cells grown at 60-80% confluency. Cultures were examined 1–2 d after plating by Ca^{2+} microfluorimetry.

Mouse primary DRG neurons-hBRIE 380i co-culture sensor system. DRG neurons were co-cultured with hBRIE 380i cells transiently transfected with the NK1R receptor. The co-culture served in an assay that could be used to characterize GLP-1 and GIP sensory nerves for their functional phenotypes. The hBRIE 380i cells, giving a SP like response, displayed no significant response to exendin-4 (Ex-4), GLP-1, GIP, capsaicin and AITC, and were grown under the DRG neuron culture conditions, were selected (sub-cloned). These cells were transiently transfected to heterologously express NK1R and found to respond to SP starting at 0.5 nM and reaching a plateau at 100 nM. They were plated on the same glass coverslip as the DRG neurons about 16 hr post DRG dispersion and plating. The co-culture was maintained in the DRG neuron culture medium at 37 °C in 5% CO_2-95% air. The hBRIE380i-NK1R cells could thus act as sensors to SP secretion from individual and adjacent neurons by displaying changes in intercellular calcium as measured by Fura-2 AM. Approximately 40 hr after DRG neuron and 16 hrs after hBRIE380i-NK1R co-culture plating, cells were preloaded with Fura-2 AM and visualized by microscopy.

Calcium mobilization assay. Calcium imaging experiments where performed as previously described (62) with some modifications. For Fura-2 AM measurement of $[Ca^{2+}]_i$ mobilization, hBRIE 380i cells and DRG neurons were incubated at 37 °C in HBSS (gibco), 0.1% BSA (Hyclone), and 20 mM HEPES (gibco) at pH 7.4 for 50 – 60 min. Fura-2 AM (Invitrogen) was used at 2 μM and supplemented with 0.01% Pluronic F-127 (w/v, Invitrogen). Coverslips were washed 3 times with 1 x Ringers and were mounted in an open chamber at 37 °C. Fluorescence of individual cells were measured

at 340 and 380 nm excitation and 510 nm emission using a Nikon Diaphot microscope. Excitation and emission were controlled by Sutter instrument LAMBDA 10-2 (UV box), video camera (PIC-III, Sutter Instrument), and a video microscopy acquisition program (Imaging Workbench 5.2). Test substances were directly added to the chamber. Each coverslip received a maximum of 6 sequential treatments, initiated with vehicle control, followed by an agonist of GLP-1R or GIPR, SP, CAP, AITC, and terminated with KCl or calcium. DRG neurons received a KCl (100 mM) treatment at termination of the experiment and hBRIE 380i cells received 10mM calcium at termination. Post experimentally, neurons were selected based on their response to KCl using ANOVA followed by Tukey's post hoc tests. They were identified as neurons if the average ratio during two 10-sec time intervals before the addition of KCl were above the number obtained using ANOVA followed by Tukey's post hoc tests. Once identified, those neurons were further selected based on their response to specific treatments by the same statistical procedure. They were deemed to be a treatment responsive if the average ratio during two 10-sec time intervals before the addition of the stimulus was above the number obtained using ANOVA followed by Tukey's post hoc tests. The same statistical procedure, ANOVA followed by Tukey's post hoc tests, was employed when comparing different treatments. Statistics were performed using Microsoft Excel. Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc test. All graphs displaying Fura-2 AM ratios have been normalized to the baseline ratio F340/F380.

Statistical analysis. All statistical tests were performed using Microsoft Excel. Values are reported as the mean ± SEM (where n = number of mice used) for $[Ca^{2+}]_i$ mobilization imaging experiments where multiple independent days of imaging were performed. A one-way single factor ANOVA followed by the Tukey's post hoc tests and the Student's t test (where appropriate) was used. Efforts were made to ensure that equal numbers of mice of each genotype were used for each experiment (where appropriate), and that treatment and control groups were of identical and near-identical size and age. Significance was labeled as follows: NS, not significant, $p \geq 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

The demonstration that MLF can induce increases in $[Ca^{2+}]_i$ in TRPV1 expressing sensory neurons from primary cultured mouse DRG has led to the possibility that sensory nerves innervating the lacteals express receptors that are activated by molecules present in the MLF and IF. Because the content of the MLF is reflective of the composition of the diet present in the intestinal lumen, we explored for the expression of receptors for molecules in the MLF known to be dependent on the dietary state of the animal, in cells within the lacteal that could induce a sensory nerve response.

GLP-1 and GIP are incretin hormones that have been established to be present in MLF in concentrations dependent on the fed state of the animal. SP has also been described

in neurons that innervate lymphatic vessels. We, therefore, examined the expression of GLP-1R on SP peptidergic nerves localized to areas exposed to IF as well as MLF. GLP-1R was found to be expressed in cells both in close and distal regions of the lacteals (Fig. 1*A*). GLP-1R and SP were found to co-localize in cells closely associated with lacteals. In contrast, cells of the sub-mucosa expressing SP were not found to express GLP-1R. TRPV1- and SP-immunoreactive cells were found in close association with the lymphatic endothelial marker LYVE-1 (Fig. 1*B* and *C*). GLP-1R was found in cells in close proximity or adjacent to LYVE-1 and within the podoplanin expressing tubular structure corresponding to endothelia of the villar lacteal (Fig. 1*B* and *C*, and Fig. 2*A*-*D*). Three -dimensional structured illumination microscopy (SIM) imaging of a lacteal rendered as a volume and isosurfaces was used to visualize structures of GLP-1R on cells within the inner face of the lumen of the lacteal (Fig. 2*A*-*D*). Volumetric rendering of SIM images show that GLP-1R was positioned in between the podoplanin expressing outer surface of the luminal facing lymphatic endothelium and the central lacteal vessel expressing LYVE-1 (Fig. 2*E*-*F*), thus placing GLP-1R in the position to come in direct contact to MLF. This is consistent with the demonstration of GLP-1R expression on dendritic cells (78), immune cells of the gut (79) and of the innate immune system (80).

Volume rendering of the SIM of villar lacteal endothelium and cells displaying TRPV1- and GLP-1R-immunoreactivity further revealed TRPV1 in cells within the lacteal (Fig. 3*A*-*C*), with distinct population of cells within the lacteal expressing only GLP-1R or TRPV1, or expressing both receptors (Fig. 3*D* and *E*). The co-localization of TRPV1 and GLP-1 in cells within the lacteal indicated potential responsiveness of TRPV1 expressing cells to GLP-1, such as those of the innate immune system as well as sensory nerves.

The co-expression of GLP1-R and TRPV1 in cells within the lacteals led us to test whether sensory neurons would be responsive to MLF from fed animals. We tested the activation of mouse primary cultured DRG neurons in response to MLF from rats taken at various time points after lipid rich Ensure™ was instilled into the duodenum. Prior to the infusion, rats that were previously cannulated at the superior mesenteric lymphatic duct were fasted overnight. Sixty minutes prior to the bolus infusion, MLF was collected for fasted values (Fig. 4*A*). MLF (from both fed and fasted rats) transiently induced $[Ca^{2+}]_i$ in about two thirds (63%) of total cultured DRG neurons (KCl sensitive) (Fig. 4*B*). Approximately one third (35%) of the neurons were MLF responsive sensory neurons, one third of which (12% of total neurons) were responsive to both capsaicin and AITC. Sensory neurons that were not responsive to MLF comprised 29% of the total neurons (Fig. 4*B*). We further explored if sensory neurons would show a differential response to MLF taken from pre- or postprandial animals. Sensory nerves exposed to MLF from fasted animals showed attenuated responses to capsaicin and AITC compared with when they were exposed to postprandial MLF (Fig. 4*C*). This indicates that MLF/IF can modulate sensory nerve response to TRPV1 and TRPA1 channel activating agents.

Given dietary induced changes in lymph, we tested if GLP-1, as an incretin hormone present in postprandial lymph, could induce a transient $[Ca^{2+}]_i$ increase in sensory

nerves. A potent GLP-1R agonist, Ex-4, at 50nM induced a transient increase in $[Ca^{2+}]_i$ in primary cultured mouse DRG sensory neurons responsive to capsaicin and AITC (Fig. 5*A*). Fifty nine percent of cultured DRG neurons only responded to Ex-4 while 12% were both Ex-4 and capsaicin responsive (Fig. 5*B*). No transient increase in $[Ca^{2+}]_i$ in response to Ex-4 in GLP-1R KO DRG neurons was observed. However, the DRG from GLP-1R KO animals contained more capsaicin responsive neurons than those from WT animals (55% vs. 38%) (Fig. 5*B*).

Our immunocytochemical data showing population of cells co-expressing GLP1-R and SP led to the question if the downstream effector response of Ex-4 in cells co-expressing GLP-1-and SP could result in the release of SP. In addition, TRPV1 and TRPA1 have been associated with the release of SP in gastric tissues and sensory neurons (81) (82). To perform this study, we developed a functional assay to determine if GLP-1 stimulation of sensory neurons expressing TRPV1 and TRPA1 could directly result in the secretion of SP.

The use of the DRG neurons as a model for GLP-1 induced activation of sensory nerves has a number of limitations when interpreting the results. One factor is that DRG neurons represent a highly heterogeneous group of neurons. Given the range of potential cell types expressing GLP-1R in the villus, it would be necessary to identify those cells whose downstream effector response, resulted from Ex-4-induced increases in transient $[Ca^{2+}]_i$, could lead to SP secretion. Studies measuring neuropeptide secretion from culture media of primary cultures of DRG neurons did not take into account the specific cell subtypes that release neuropeptides. In addition, the assay methodology was often not sufficiently sensitive to secretory products in the media whose concentrations fall below the detection threshold. Similarly, detection of transient changes in $[Ca^{2+}]_i$ of a single sensory neuron does not directly translate to the secretion of peptides from that neuron.

Therefore, we developed a system whereby non-tumorigenic rat mucosal epithelial cells (hBRIE 380i cells) transfected to heterologously express the SP NK1R could be cultured with primary dispersed mouse DRG neurons. These hBRIE 380i-NK1R cells could act as SP sensors that would respond to the SP released from individual and adjacent neurons by displaying changes in $[Ca^{2+}]_i$ (measured using Fura-2 AM) (Fig. 6). Non-tumorigenic hBRIE 380i cells that displayed no endogenous responses to Ex-4, GLP-1, GIP, capsaicin, AITC, and SP (Fig. 6*A*) were selected (subcloned) to grow under DRG neurons culture conditions. These cells were transiently transfected to heterologously express NK1R and found to respond to SP starting at 0.5nM and approaching a plateau at 100nM (Fig. 6*B*). Dispersed DRG and hBRIE 380i-NK1R co-cultures were preloaded with Fura-2 AM and visualized by microscopy for changes in $[Ca^{2+}]_i$ in response to a variety of stimulants. Fig. 6*D-F* is a representative response plot using the SP sensor bioassay. Transient increase in $[Ca^{2+}]_i$ in response to 50nM Ex-4 was found in both nerves responsive to AITC and capsaicin that also resulted in SP-sensor response (SPR) (Fig. 6*F*). These transient increases in $[Ca^{2+}]_i$ were also found in neurons that did not display SPR (Fig. 6*F*) indicating that GLP-1 has effects on peptidergic sensory and non-sensory neurons. The Ex-4 induced SPR suggests that a

number of GLP-1 effects could be mediated through SP and other neuropeptides secreted from sensory neurons.

Ex-4 induced a transient increase in $[Ca^{2+}]_i$ in DRG sensory neurons. A significant increase in the SPR was observed in sensors adjacent to those neurons (Fig. 7*A*). Consistent with the observations of the increased number of DRG sensory neurons responding to capsaicin from the GLP-1R KO animals, we observed an enhancement of the transient increase in $[Ca^{2+}]_i$ corresponding to increases in SPR with capsaicin treatment in GLP-R KO neurons (Fig. 7*B*). This suggests that GLP-1 attenuates capsaicin induced neuropeptide secretion. This is consistent with the increase in SPR to Ex-4 that was observed in TRPV1 KO DRG neurons (Fig. 8*A*). However, attenuation of the transient increase in $[Ca^{2+}]_i$ in DRG neurons that also parallels attenuation in SP secretion in the presence of Ex-4 remains in the TRPA1 KO DRG neurons (Fig. 8*B*). No significant difference in the transient increase in $[Ca^{2+}]_i$ was observed in DRG neurons $[Ca^{2+}]_i$ response or in the SP-sensor response in sensory neurons from TRPA1/TRPV1 double KO animals. Thus Ex-4 induced SP secretion is dependent on the expression of either or both the TRPA1 and TRPV1 channels. (Fig. 9).

Given that GIP is also an incretin that has been found in postprandial MLF, we examined if GIP, like GLP-1, could also result in a SPR. GIP responsive neurons were found to induce SP secretion from GIP responsive neurons (Fig. 10). However unlike GLP-1, GIP was found to enhance rather than attenuate capsaicin induced SP release from peptidergic sensory neurons.

Discussion

In these studies, we present a pathway whereby the contents of the IF can be monitored by the peripheral tissues and central nervous system (CNS) through the sensory nerves associated with the lymphatic vasculature. These sensory nerves represent an extra-vagal route for the CNS to respond to molecules, present in the IF and associated with tissue ECM, through local lymphatic vessels. Traditionally the lymphatic system has been viewed as a passive network of capillaries and collecting vessels that plays a critical role in maintaining interstitial pressure, the transport of molecules such as lipids, antigen presentation, and returning plasma extravasated proteins to the circulation (83) (84).

Recent attention has been given to the secretory products of cells that comprise the IF for their potential ability to initiate biological responses, such as immune function and tumor growth. The composition of the MLF is a reflection of interstitial fluid and is subject to similar endogenous determinates, such as hydrostatic pressure and transcapillary exchange of proteins in the blood (43). Additionally, MLF is composed of molecules from exogenous sources, such as from diet in the intestinal lumen. Molecules of the IF could both be monitored by the CNS and have their biological activity coordinated to yield physiological responses through peripheral sensory nerves. The lymphatic microvasculature provides a point of convergence for molecules from local pools of IF, to activate sensory nerves associated with those vessels or capillaries.

The characterization of the lysophosphatidic acid receptor LPA5 provided evidence that receptors on sensory nerves were responsive to dietary induced changes of lymph/IF contents, and brought forth a mechanism for direct activation of primary sensory neurons in response to intestinal contents and the subsequent release and accumulation of peptides into the IF/MLF (63) (64). This led to the possibility that GPCRs, like LPA5, expressed in sensory nerves and found in peripheral lymphatic vessels could also perform a similar function in response to molecules in the lymphatic fluid derived from the interstitium. Additionally, the transient increase in $[Ca^{2+}]_i$ observed in response to MLF was dependent on the fed state of the animal which indicates that the composition of MLF/IF is also a reflection of exogenous sources, such as from the intestinal lumen. Similar to the mucosal lacteals, the medial layer of peripheral lymphatic vessel walls is also richly innervated with SP-, CGRP- and other peptide-containing nerves (85) (86). Therefore, sensory nerves of the lymphatic vasculature could provide a conduit for the afferent signaling of bioactive molecules in IF accumulated in the mesenteric lymph vessels through the proposed neurolymphocrine system (62). This is exemplified in the diet dependent induction of the transient increases in $[Ca^{2+}]_i$ that we observed in the sensory nerves responsive to capsaicin and AITC upon exposure to MLF.

It was previously reported that GLP-1 did not induce transient increases in $[Ca^{2+}]_i$ in primary cultured DRG sensory neurons (87). In that study capsaicin sensitivity was not affected by acute treatment with 100 nM of Ex-4 or GLP-1, compared to controls (12 neurons). Enhanced ATP signaling in the presence of Ex-4 led to the conclusion that nociceptive capsaicin / heat pain receptor TRPV1 was not affected by acute application of GLP-1, that was taken as an indication that the GLP-1R did not participate in signaling involving TRPV1 (87).

However, in our study we observed significant transient increases in $[Ca^{2+}]_i$ in response to both GLP-1R and GIP-R activation, which is consistent with the transient increases in $[Ca^{2+}]_i$ mediated by $G\alpha_q$ pathways, and by $G\alpha_s$, observed for insulin induced secretion by activation of GLP-1R (88-91). This exemplifies the importance of linking signaling pathways in the context of a functional effector response. This is especially true for the B family GPCR's, such as GLP-1R and GIPR, which can be pleiotropically coupled with multiple signaling pathways important for receptor function (92).

Conclusions derived from studies using primary culture DRG models need to take into consideration *in vitro* conditions that are bias for particular subpopulations compared to the heterogeneity of DRG neurons *in vivo*. For example, neuron populations in culture can be induced to become peptidergic in the presence of nerve growth factor (93). The labeling of specific subpopulations of DRG, and determining their corresponding function remains challenging. Efforts have been made to determine molecular markers and the transcriptional profiles of DRG neurons, both at whole population and single cell levels in an effort to determine their modality and their activators (94). This has allowed the identification of a greater number of DRG neurons beyond the general classification of nerve fiber subtypes (Aα, Aβ, Aδ and C fibers) (95). In our study, we characterized DRG neurons based on their ability to secrete SP in response to GLP-1 and GIP, thus,

establishing a functional phenotype for cell subpopulations. The co-culture sensing system allowed us to examine subpopulations of DRG neurons that when activated by GLP-1 and GIP induce a transient increase in $[Ca^{2+}]_i$ that results in SP release. These cells can be contrasted to those subpopulations of neurons that responded to GLP-1 and GIP with a transient increase in $[Ca^{2+}]_i$ which do not result in SP release. The characterization of these subpopulations of neurons could elucidate further biological effects of incretins on non-peptidergic neurons.

Both TRPV1 and TRPA1 are members of a subset of transient receptor potential (TRP) proteins which belong to the superfamily of cation channels that have central roles as noxious and chemo sensors (96). The expression of TRPV1 in cells adjacent to and within the lacteals, such as macrophages, neutrophils, and mast cells (70), and sensory nerves associated with lacteals, positions these cells as potential signal transducers that respond to chemical stimuli from the IF of local tissue pools.

The release of SP from gastrointestinal tissues has been closely correlated to the expression and activation of TRPV1 and TRPA1 from cells of those tissues (81,82,97). Our demonstration that Ex-4 induced SP secretion is blocked only in TRPV1 and TRPA1 double KO neurons supports the suggestion that TRPA1 and TRPV1 co-expression on DRG neurons could lead to the 2 channels interacting and modulating each other's activities (55). This might be exemplified by an accumulated transient increase of $[Ca^{2+}]_i$ leading to SP secretion. This regulation could have significance physiological importance for GLP-1 as a modulator of TRP proteins on peptidergic neurons such as those that secrete SP.

SP has a wide range of activities from acting in neuromodulation, neuronal integrity, sensory perception, and pain, to regulation of movement, respiration, inflammation and depression (98). GIPR and GLP-1R induced increases in $[Ca^{2+}]_i$ in sensory nerves result in release of SP from individual nerves is of particular interest because SP shares many biological effects with GLP-1 amide which includes vasodilation (99,100), cell growth (101,102), and pancreatic neuroprotection (103,104) (105). This raises the likelihood of synergy between GIP/GLP-1 and SP on end organ response, for example, the modulation of inflammatory responses and blood pressure.

Our data suggest that GLP-1, by itself, could act as an inflammatory mediator by activating sensory nerves via TRPV1 and/or TRPA1 to release SP. However, under conditions where sensory neurons are presented to GLP-1 prior to capsaicin stimulation, the capsaicin-induced inflammatory response resulting in SP release is attenuated. This supports not only an interplay between TRPV1 and TRIPA1 on $[Ca^{2+}]_i$ and nociceptor function(106,107), but also modulation of that interplay by factors whose concentrations fluctuate in the IF/MLF such as GLP-1

The activation of NK1R by SP could be a pathway for TRPV1 to induce inflammation. Both pro and anti-inflammatory effects of GLP-1 have been described to be tissue specific (91). GLP-1 can inhibit the recruitment and activation of macrophages, and

reduce the release of pro-inflammatory mediators in monocytes (108). However, in the brain, the presence of GLP-1R results in increased IL-6 expression (109), indicating a potential pro-inflammatory effect.

Our data indicate that the degree of GLP-1 induced SP release is dependent on the timing and exposure of GLP1 on sensory nerves expressing TRPV1 and/or TRPA1 channels. In mouse primary cultured DRG neurons, GLP1 induces SP release in both TRPV1 and TRPA1 expressing neurons independent of capsaicin and AITC. Capsaicin and AITC alone induce SP release that is significantly elevated over that of GLP-1 alone. However, the prior exposure of GLP-1 on neurons significantly attenuates the levels of released SP in response to subsequent exposure to capsaicin. This attenuation of SP release was not observed with DRG neurons similarly treated with AITC, which could indicate independent inhibitory pathways initiated by GLP-1R activation of TRPV1 that is not linked to TRPA1. However, DRG neurons from TRPV1 and TRPA1 double KO mice did not display Ex-4 induced SP secretion, thus, indicating a shared signaling pathways that leads to the release of SP. SP secretion resulting from GLP-1/GIP activation of sensory nerves could provide a pathway for molecules found in the IF and/or lacteals to modulate the innate immune function or inflammation in response to diet dependent signals.

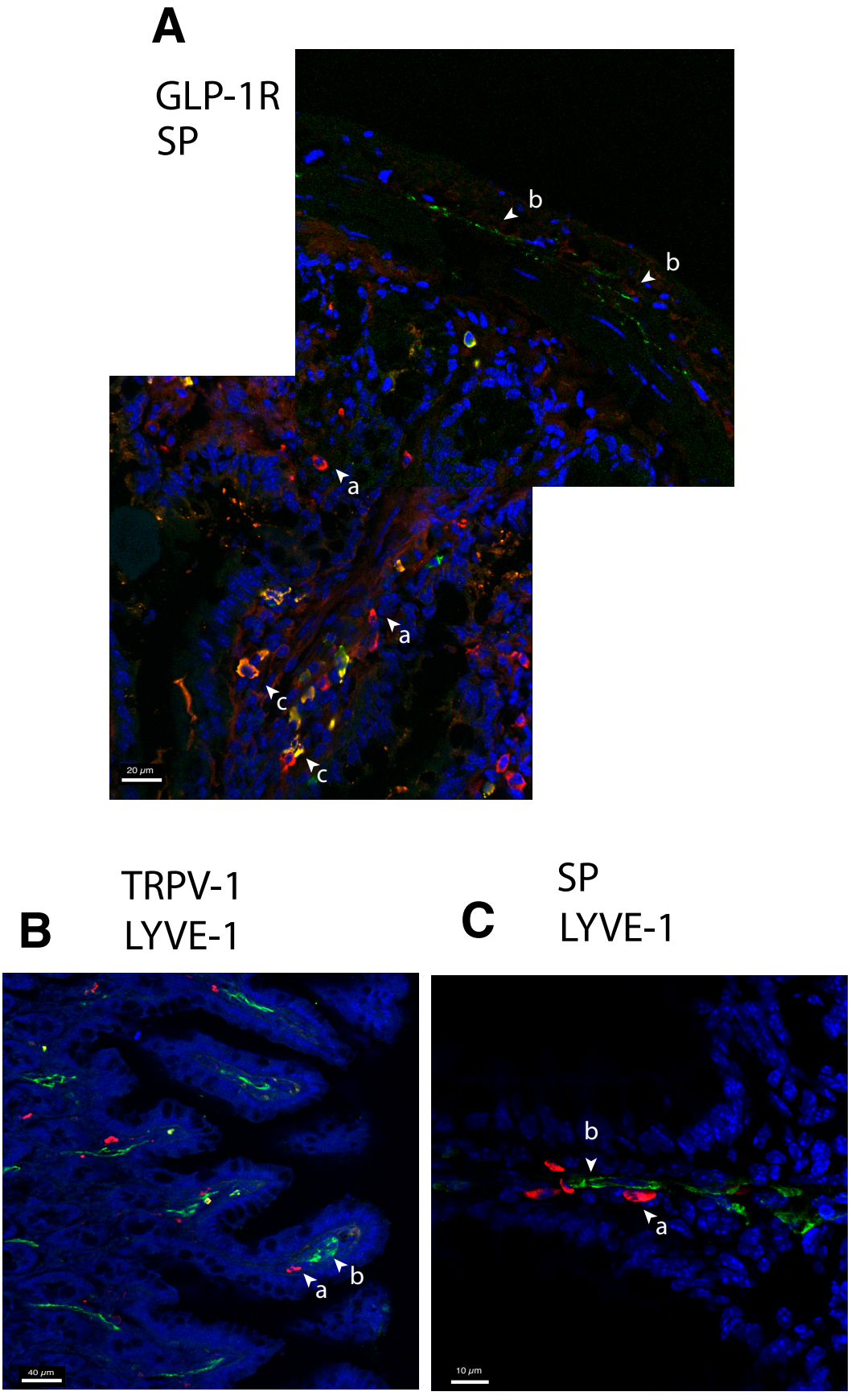
SP is present in all peripheral sensory nerves particularly those associated with blood vessels. Activation of NK1 has been shown to induce vasodilation and reduce blood pressure (110). GLP-1 has also been shown to increase blood flow and decrease blood pressure. GLP-1 has been implicated in having a direct effect in vasodilation via endothelial nitric oxide synthase (111). The blood pressure lowering effects have been observed in mouse models of hypertension (112). The potential modulation of blood vessel dilation by GLP-1, through the release of SP sensory nerves associated with blood vessels, expands the mode of action of GLP-1 as having a “local effector function.” A term first used for peripheral endings of afferent nerves and the adjacent tissues that organize immediate response to stimuli (110). In our model the dietary induced release of GLP-1, resulting in its accumulation in the MLF and IF, would induce vasodilation and increase blood flow via SP release from sensory nerves in close association with capillary endothelium. This would be independent of its role on glycemic control yet modulated by dietary intake.

Apart from our newly described effects of GLP-1 and GIP via the release of SP from sensory neurons, our study further broadens the mode of action as of GLP-1 and GIP as incretins. While the activation of afferent nerves by GLP-1 and GIP has significant effects on pancreatic response through canonical pathways such as vagal afferents, its effects on the pancreas and islet responses could also be exerted through sensory pathways involving spinal nerves. Classically, neuro modulation of pancreatic responses is through vagal afferent neurons. However, it has been reported that up to 70% of retrogradely labeled small to medium pancreatic primary afferent neurons (in both the thoracolumbar DRG and the nodose ganglia) show TRPV1 immunoreactivity (113). Hence, the majority of pancreatic primary sensory neurons express TRPV1. It

has been demonstrated that sensory nerves of the DRG, such as those containing calcitonin gene related peptide (CGRP), have cell bodies that project to the distal intestine (114). Thus, MLF induced local release of SP from sensory neurons of DRG suggests the potential of a similar pathway whereby GLP-1 and GIP from lacteals could exert their effects on sensory nerves of pancreatic islets through canonical pathways, such as vagal afferents, as well as sensory pathways involving spinal nerves and the CNS (115) in response to exogenous and endogenous stimuli.

The presence of GLP-1 and GIP in the ML/IF and their induced release of SP expand the effector response of these incretin hormones through the sensory nerve secretion of SP. The mode of action of GLP-1 and GIP via SP from sensory neurons is not likely confined the release of this neuropeptide. At least 12 neuropeptides have been reported to be present in TRPV1 positive sensory neurons (61,116). These include somatostatin, vasoactive intestinal polypeptide, cholecystokinin, galanin and corticotrophin-releasing factor, all of which have established systemic effects. The proposition that multiple neuropeptides can be simultaneously released from TRPV1 activated sensory nerves further expands the potential mode of action of GLP-1 and GIP beyond the effects of SP. The local MLF/IF induced neuropeptides release from sensory nerves suggests the likelihood of a shared pathway for molecules found in the IF and MLF, such as GLP-1 and GIP, that can exert their effects on peripheral tissues as well as the CNS (115) in a pathway autonomous of the vascular circulation through a neurolymphocrine system.

Figures and Legends:


A
GLP-1R
SP
b
b
a
a
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c
20 μm
B
TRPV-1
LYVE-1
a
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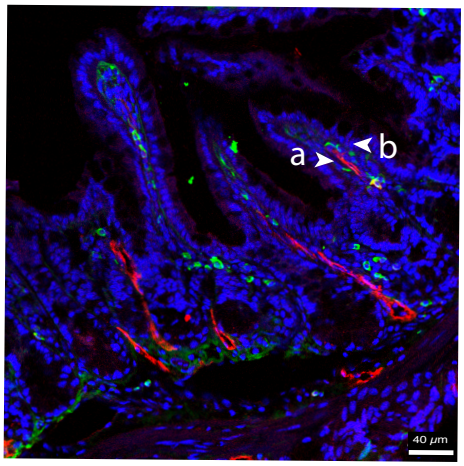
SP
LYVE-1
b
a
10 μm

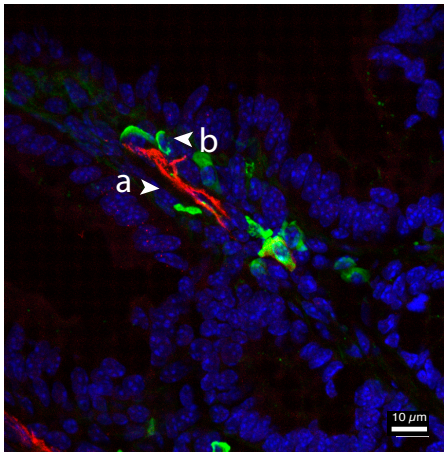
Fig. 1.

Immunocytochemical localization of GLP-1R, SP and TRPV1 in mouse distal small-intestinal lacteals and in nerve fibers. *A:* A low magnification confocal image montage of microvilli demonstrating GLP-1R (red, rhodamine labeled) on cells along the length of mucosal villus (arrows a). GLP-1R and SP (green, FITC labeled) cells were found to co-localize in the mid villar region (yellow, arrows c), in contrast to the submucosa where SP was detected in nerve fibers void of GLP-1R (arrows b). *B:* TRPV1 (arrow a) was found in cells in regions along the center of the villus in proximity to LYVE-1 (arrow b) of the lacteal endothelia. *C:* SP (arrows a) was observed in close association with LYVE-1 of the lymphatic endothelia. DAPI-stained nuclei are blue. The Object Pearson and Manders co-localization were determined using HuygensPro software.

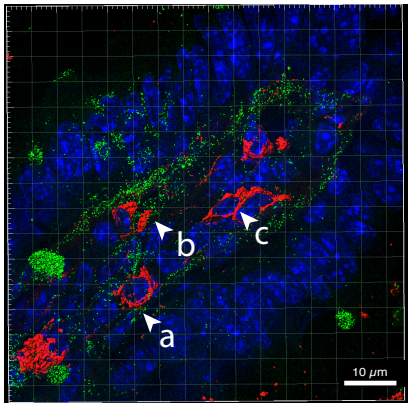
A GLP-1R
LYVE-1



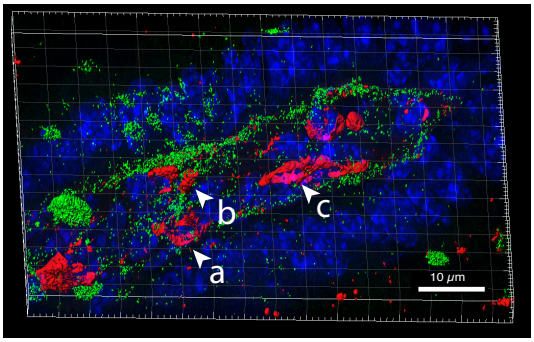
B GLP-1R
LYVE-1



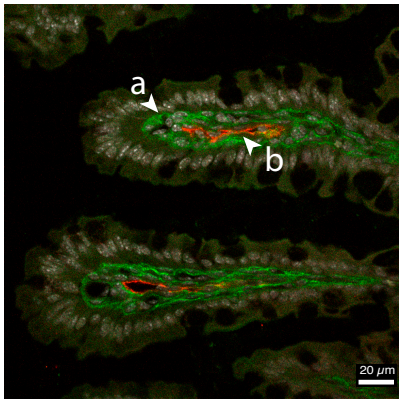
C GLP-1R
podoplanin



D GLP-1R
podoplanin



E LYVE-1
podoplanin



F LYVE-1
podoplanin

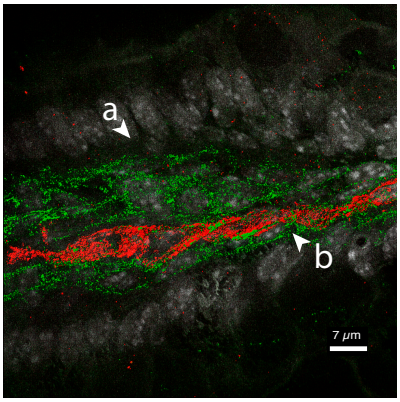
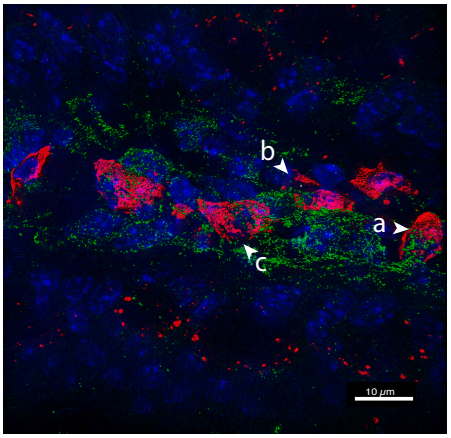


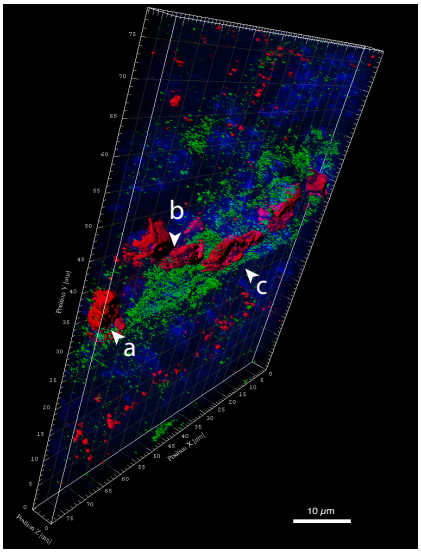
Fig. 2.

Immunocytochemical localization of GLP-1R within the inner luminal surface of the villar lacteal endothelium. *A* and *B:* Maximum intensity projection villar LYVE-1 (rhodamine) is shown localized primarily to an inner central area of the lacteal endothelium (arrow a) compared to the pattern of expression of GLP-1R (FITC, arrow b). *C:* 3D-SIM imaging of a lacteal rendered as a volume (Imaris). Isosurfaces were used to visualize structures of GLP-1R (red, rhodamine) cells (arrows a,b,c) surrounded by podoplanin endothelium (green, FITC). *D:* Rotational projection shows GLP-1R on cell surfaces (arrows a,b,c) within the lacteal endothelial tubular structure forming the lacteal outlined by podoplanin. *E:* Confocal microscopy imaging of a mucosal villus demonstrating differential distribution of podoplanin (arrow a) and LYVE-1 (arrow b) on the lacteal endothelium. *F:* Volumetric rendering of SIM images show expression of the endothelial markers (arrows a,b) in a diffuse pattern of podoplanin on the outer surface of the endothelium (arrow a) while LYVE-1 is distributed as in a more confined area within the central region within the lacteal (arrow b). Images are representative of a minimum of five fields viewed of a minimum of 5 replicate samples of tissue sections per animal (n=6) with at least two technical replicates per set of samples.

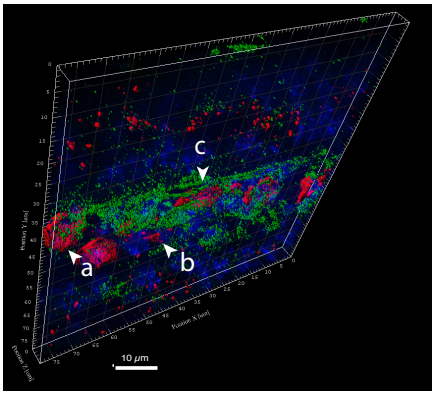
A TRPV-1
podoplanin



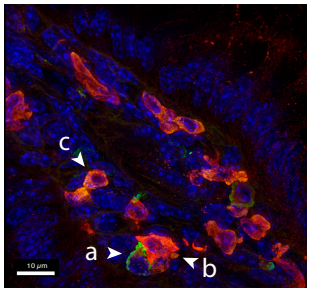
B TRPV-1
podoplanin



C TRPV-1
podoplanin



D GLP-1R
TRPV-1



E GLP-1R
TRPV-1

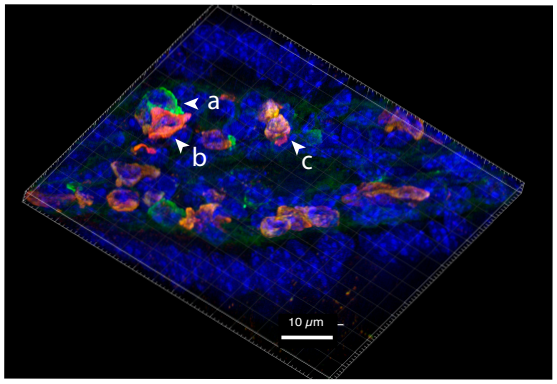
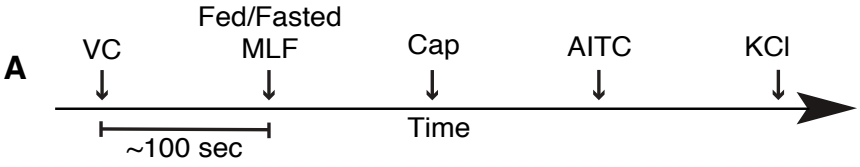
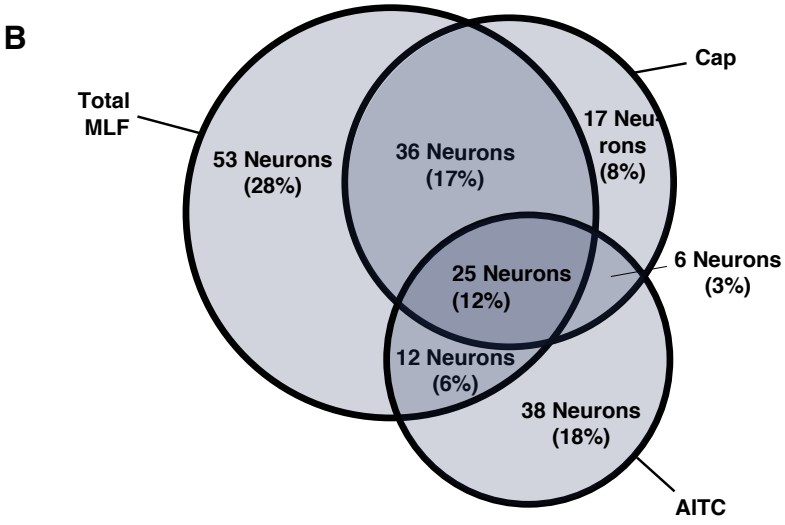


Fig. 3.

Volume rendering of SIM of villar lacteal endothelium and cells displaying TRPV1 and GLP-1R. *A-C:* Rotated projections of SIM image stack from panel *A* are displayed in panels *B* and *C*. Isosurface renderings of structures showing lacteal with TRPV1 (rhodamine) cells (arrows a,b,c) surrounded by podoplanin endothelium (FITC). Projection in panel *B* shows cells (arrows a,b,c) of panel *A* with no podoplanin covering the outer surface of the cells from the inner (luminal) side of the lacteal vessels. *C:* 180° rotation of isosurface rendered in panel *B* revealed masking of TRPV1 fluorescence on cells (arrows a,b,c) by podoplanin indicating cells lie within the lacteal endothelial vessel. *D:* Maximum intensity projection of GLP-1R (green, FITC) in cells that were found to be adjacent (arrow a) to cells displaying TRPV1 (arrow b) or to co-localize (arrow c) with cells expressing TRPV1 (arrows c). *E:* is a similar projection of cells (arrows a,b,c) from panel *D* showing the same relationship. Images are representative of a minimum of five fields viewed per replicate with at least two technical replicates and the experiment was conducted in at least three biological replicates.


A
VC
Fed/Fasted
MLF
Cap
AITC
KCl
Time
~100 sec


B
Total
MLF
Cap
53 Neurons
(28%)
36 Neurons
(17%)
17 Neu-
rons
(8%)
25 Neurons
(12%)
6 Neurons
(3%)
12 Neurons
(6%)
38 Neurons
(18%)
AITC

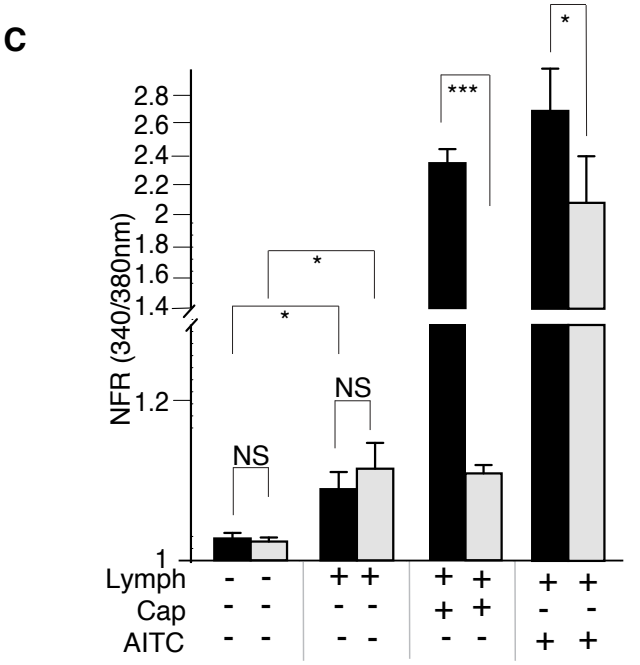
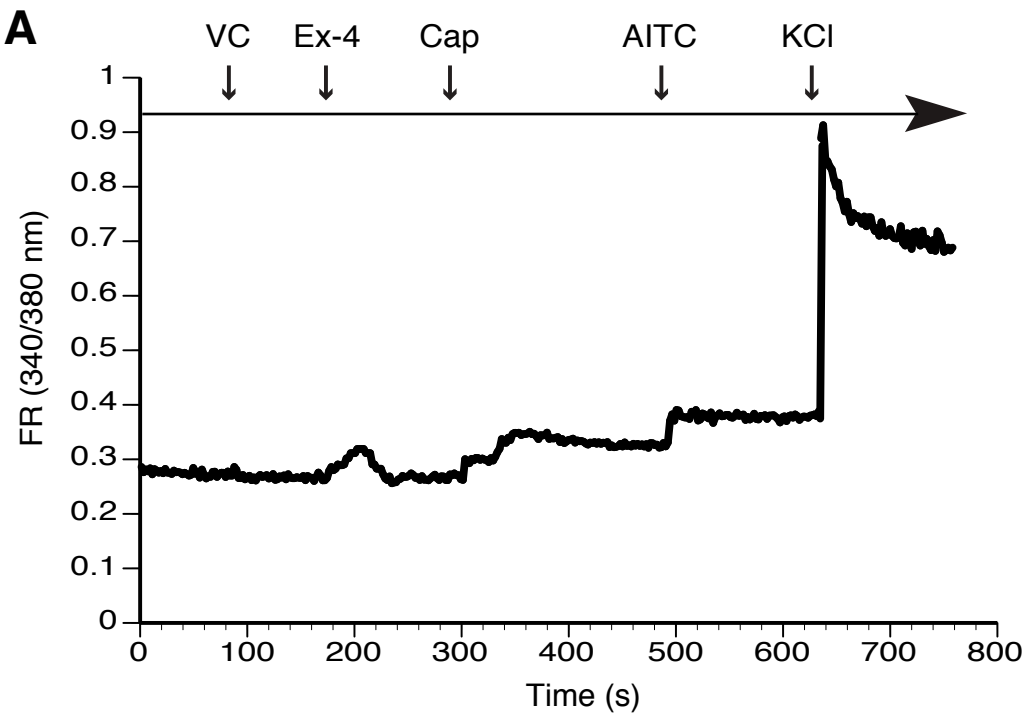

C
NFR (340/380nm)
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
NS
NS
*
*
***
*
Lymph - - + + + + + +
Cap - - - - + + - -
AITC - - - - - - + +

Fig. 4.

Mesenteric lymphatic fluid induces a transient increase in $[Ca^{2+}]_i$ in primary cultured mouse dorsal root ganglia (DRG) neurons. *A*: Time line used for Fura-2 AM calcium imaging assay depicting the approximate time intervals (about every 100 sec apart) with the respective treatments, vehicle control (VC, 1x Ringers), followed by fed and/or fasted MLF (at 120 min after food intake for fed, 0 min for fasted), capsaicin (Cap, 1μM), allyl isothiocyanate (AITC, 1μM) and KCl (100mM). *B*: $[Ca^{2+}]_i$ assay was done on mouse primary cultured DRG neurons as described in figure 6. They were exposed to total MLF (1:50 dilution) taken from fed and fasted rats combined (n=3). Sixty-three percent of the total neurons responded to MLF, 28% were not sensory nerves. Seventeen percent and 6% of the MLF responsive neurons were sensory, Cap and AITC, respectively. Sub-populations of non-MLF responsive neurons were only responsive to Cap (8 %) or AITC (18 %). A similar percentage of neurons respond to either Cap or AITC, 40% and 39%, respectively. *C*: Cultured DRG neurons presented with lymph from fasted animals, (□) showed attenuation in the transient increases in $[Ca^{2+}]_i$ in response to both Cap and AITC compared to lymph from fed animals (■). Bars are means SE (n=3). NS, not significant, $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.


A
VC
Ex-4
Cap
AITC
KCl
FR (340/380 nm)
Time (s)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
100
200
300
400
500
600
700
800

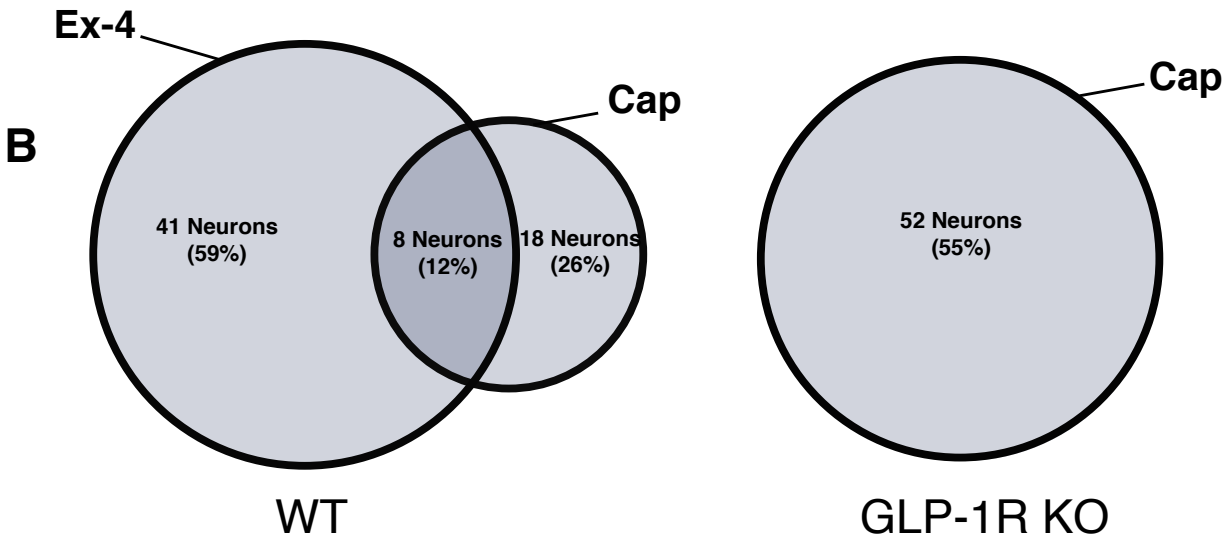

B
Ex-4
Cap
41 Neurons
(59%)
8 Neurons
(12%)
18 Neurons
(26%)
WT
Cap
52 Neurons
(55%)
GLP-1R KO

Fig. 5.

Ex-4 induces a transient increase in $[Ca^{2+}]_i$ in primary cultured DRG neurons. *A:* Tracing of a single mouse primary cultured DRG neuron treated sequentially with VC (1x Ringers), 50 nM Ex-4, Cap, AITC, and KCl; as described in Fig. 4. *B:* The Venn diagram depicts a higher percentage of neurons responding to only Cap treatment in the GLP-1R KO compared to WT. Approximately half of the neurons responded to both Cap and Ex-4 in the WT animals.

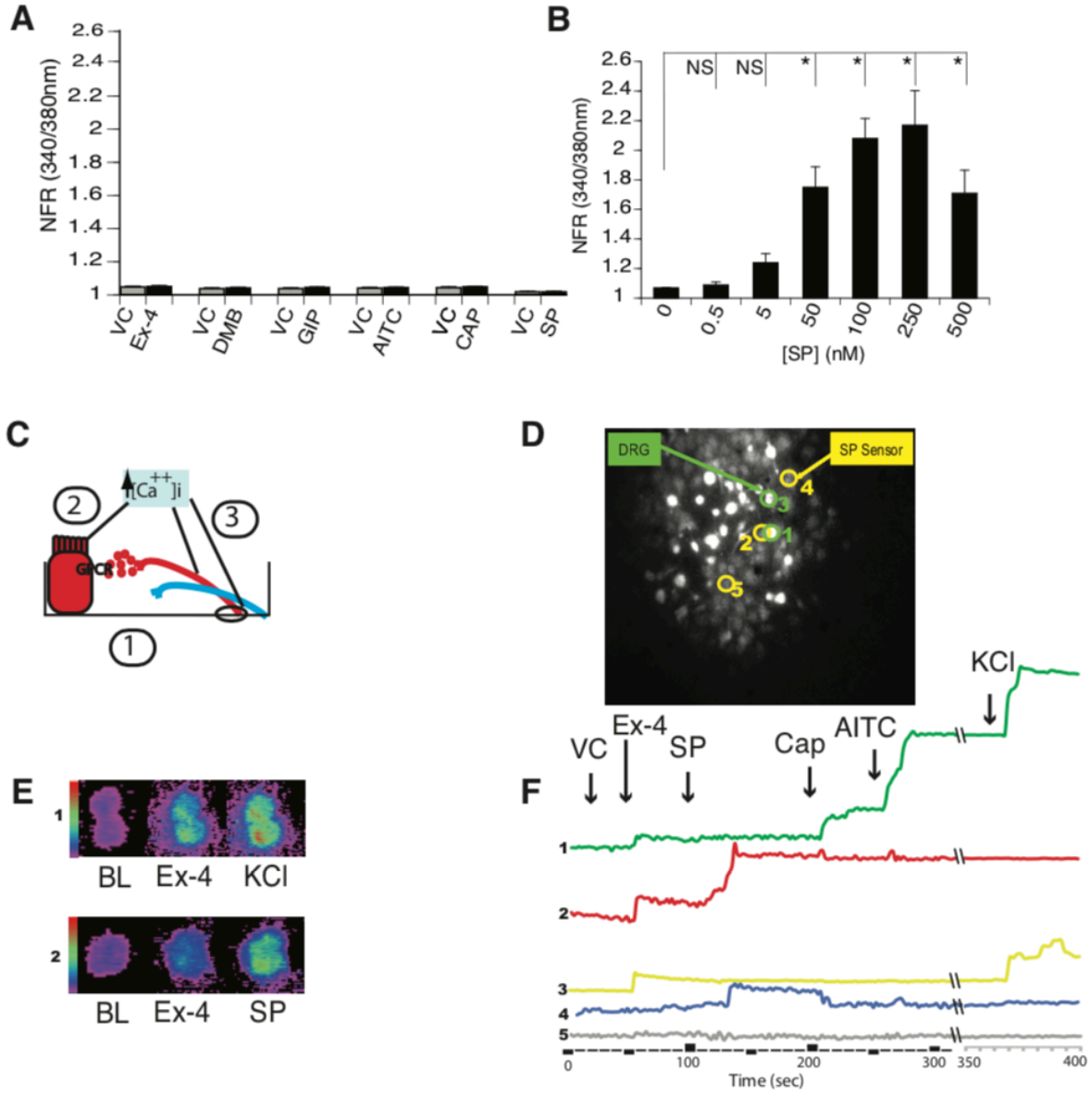

A
NFR (340/380nm)
VC
Ex-4
DMB
GIP
AITC
CAP
SP
B
NS
NS
[SP] (nM)
C
[Ca++]i
D
DRG
SP Sensor
E
BL
Ex-4
KCl
SP
F
VC
Ex-4
SP
Cap
AITC
KCl
Time (sec)

Fig. 6.

The DRG neurons and hBRIE 380i cells co-culture bioassay for determining a SP functional phenotype of individual sensory nerves. *A:* hBRIE 380i cell subclones did not show an increase in $[Ca^{2+}]_i$ in response to either Ex-4 (50 nm), GLP-1 (50 nm), GIP (50 nm), AITC (1uM), or Cap (1uM), or to SP (250 nM), black bars (■) compared with the vehicle control (VC). Bars are means ± SE (n=3). NS, not significant, $p \geq 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *B:* The hBRIE 380i cells heterologously expressing the NK1R receptor were stimulated using different concentrations of SP. The SP-sensors responded to SP in a range from 0.5 nM to 250 nM. Bars are means ± SE (n=3). NS, not significant, $p \geq 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *C:* Diagram illustrating sensor cell model. (1) Dispersed DRG neurons were plated on 18 mm round borosilicate glass coverslips and co-cultured with SP sensors. Fifty to sixty min prior to microscopy, cells were loaded with 2 uM Fura-2 AM. After preloading with Fura-2 AM, cultured cells were treated with vehicle control (VC) followed by Ex-4 and lastly with SP to identify sensor cells, Cap to identify cells expressing transient receptor TRPV1, AITC to identify cells with TRPA1, and KCl to identify nerves. Activation was determined by changes in intracellular calcium (2 and 3). Sensor cells (2) were activated by secreted SP from adjacent nerves. *D* and *F*: Example of the bioassay for SP secreted from single neurons using the co culture of neuronal cells with SP sensor cells. Cell 1 was a neuron (KCl responsive) that released SP in response to Ex-4 expressing both TRPA1 and TRPV1 channels. The SP response was detected by sensor cell 2 in the presence of Ex-4 (red). Cell 3 was a nerve that responded to Ex-4, but did not release SP as indicated by sensor cell 4, and was not responsive to TRPA1 and TRPV1 agonists. Cell 5 was a non-transfected hBRIE 380i cell. *E:* The range of detection in the transient $[Ca^{2+}]_i$ depicted calorimetrically for in the DRG neurons (panel 1) and SP-sensors (panel 2) when treated with Ex-4 and SP.

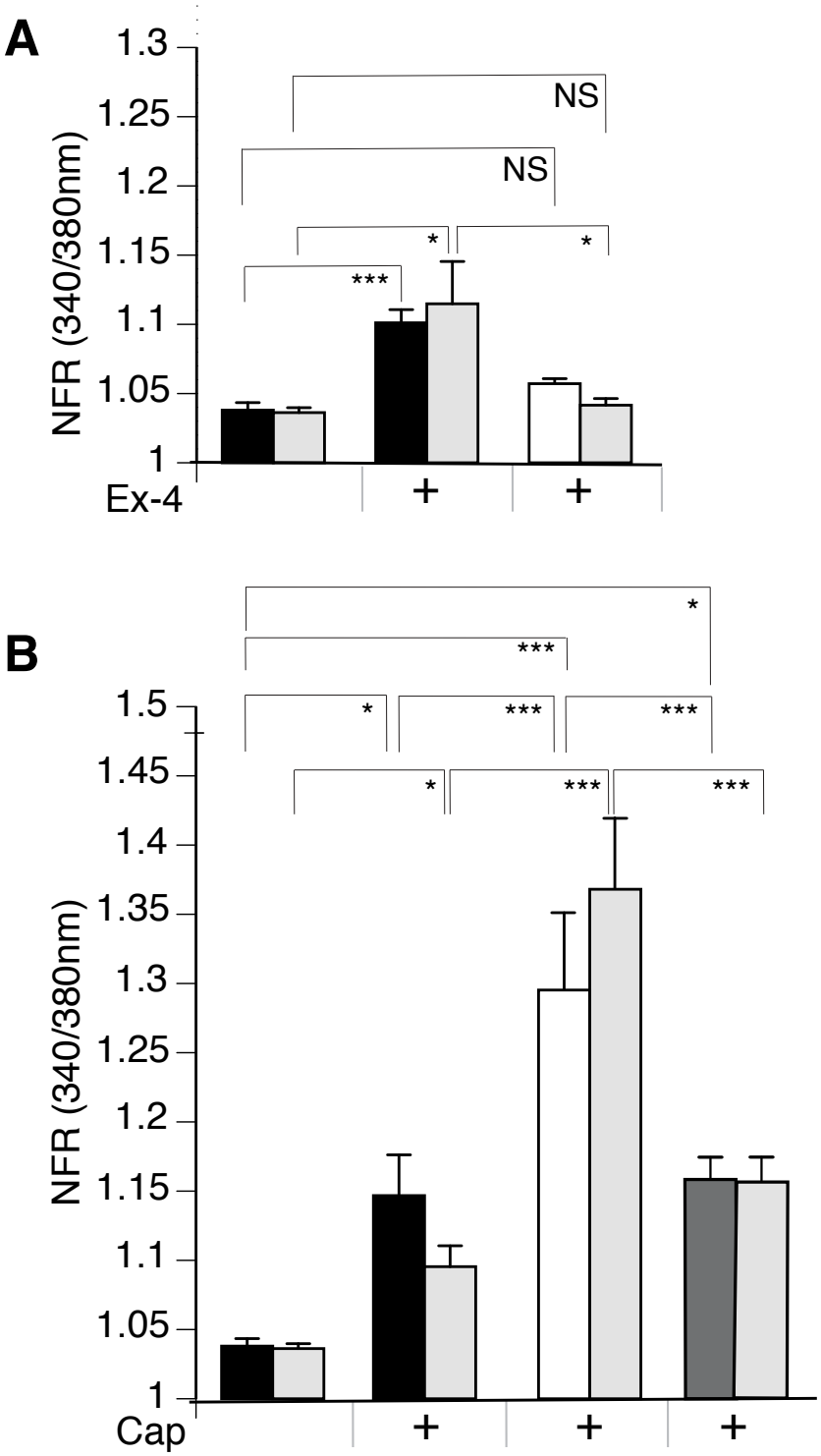
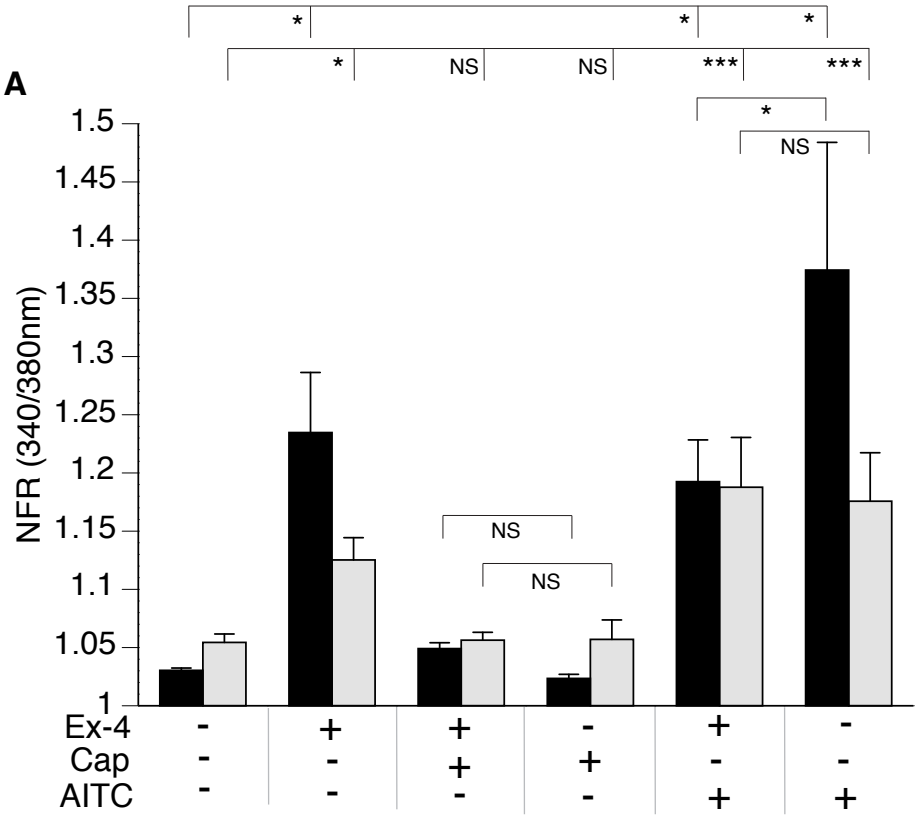

A
1.3
1.25
1.2
1.15
1.1
1.05
1
NFR (340/380nm)
NS
NS
*
*
***
Ex-4
+
+
B
*
***
*
***
***
*
***
***
1.5
1.45
1.4
1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
NFR (340/380nm)
Cap
+
+
+

Fig. 7.

Ex-4 treatment of DRG neurons significantly induces release of SP, and attenuates SP release in response to Cap. Mouse primary cultured DRG neurons from WT (■) and GLP-1R KO ( ) animals were co-cultured with SP-sensors (□) and exposed to 50 nM Ex-4, followed by Cap and KCl. Concentrations of the treatments were as described in Fig. 6. For GLP-1R rescue, DRG from GLP-1R KO animals were transiently transfected with GLP-1R. *A:* Ex-4 induced a significant transient increase in the $[Ca^{2+}]_i$ in the WT (■), but not in GLP-1R KO ( ). The sensor response corresponded to the DRG neuron calcium response, showing significance in the WT and no significance in the GLP-1R KO. *B:* Cap, in the presence of Ex-4, significantly stimulated SP response in the WT and GLP-1R KO rescue ( ) but not in the GLP-1R KO. NS, not significant, $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.


A
NFR (340/380nm)
1.5
1.45
1.4
1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
*
*
*
*
NS
NS
***
***
*
NS
NS
NS
Ex-4 - + + - + -
Cap - - + + - -
AITC - - - - + +

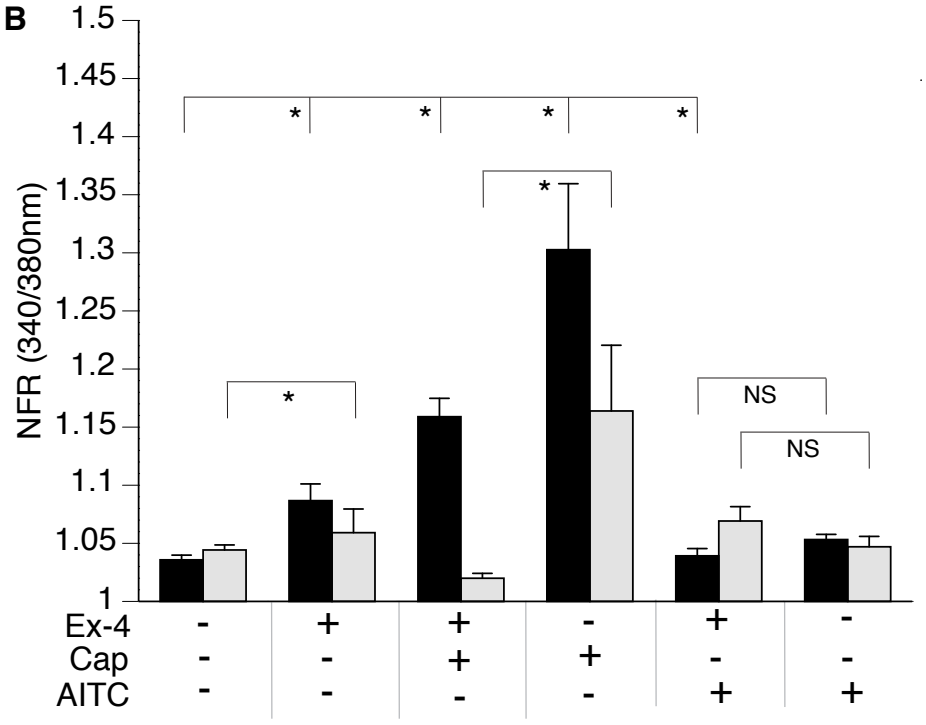

B
NFR (340/380nm)
1.5
1.45
1.4
1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
*
*
*
*
*
*
NS
NS
Ex-4 - + + - + -
Cap - - + + - -
AITC - - - - + +

Fig. 8.

Ex-4 induced SP release from DRG neurons occurs in sensory neurons that express TRPV1 or TRPA1 channels. DRG from TRPV1 KO (*A*) and TRPA1 KO (*B*) animals were co-cultured with SP sensors (□) and exposed to 50 nM Ex-4, followed by Cap, AITC, and KCl. Concentrations of the treatments were as described in Fig.6. *A*: TRPV1 KO neurons (■) still produces a significant increase in Ex-4 calcium response and also a corresponding SP-sensor (□) response. Cap treatment, however, does not result in significant difference in either DRG neurons or SP-sensor $[Ca^{2+}]_i$. An attenuation in the transient increase in the $[Ca^{2+}]_i$ of DRG neurons calcium, but not in SP, is observed with AITC treatment. *B*: TRPA1 KO neurons (■) also show a transient increase in the $[Ca^{2+}]_i$ in response to Ex-4 which corresponds to SP secretion(□).

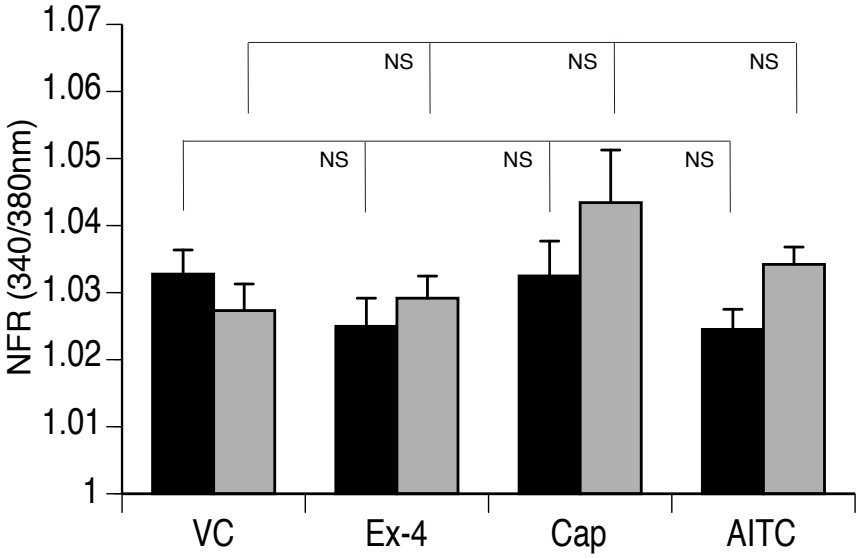

1.07
1.06
1.05
1.04
1.03
1.02
1.01
1
NFR (340/380nm)
NS
NS
NS
NS
NS
NS
VC
Ex-4
Cap
AITC

Fig. 9.

Ex-4 induced SP release does not occur in sensory neurons from TRPA1/TRPV1 double KO DRG. Mouse primary cultured DRG neurons (■) from TRPA1/TRPV1 double KO animals were co-cultured with SP-sensors (□) and exposed to 50 nM Ex-4, followed by Cap, AITC and KCl. Concentrations of the treatments were as described in Fig. 6.

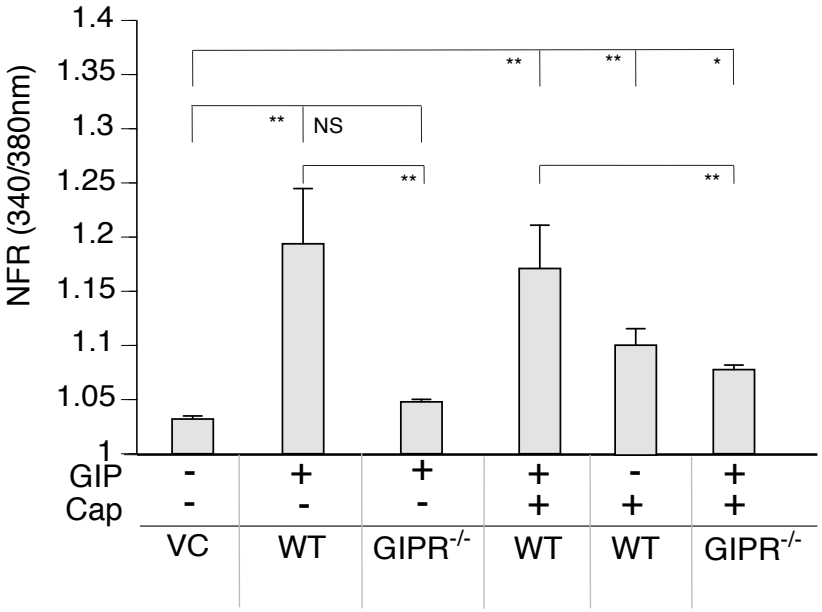

NFR (340/380nm)
1.4
1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
**
**
*
**
NS
**
**
GIP - + + + - +
Cap - - - + + +
VC
WT
GIPR-/-
WT
WT
GIPR-/-

Fig. 10.

GIP induces SP release from capsaicin responsive DRG sensory neurons. The graph depicts SP-sensor (□) response. Mouse primary cultured DRG neurons from WT and GIP KO animals were co-cultured with SP sensors and exposed to 50 nM GIP. GIP induced an increased calcium response in SP-sensors adjacent to Cap responsive sensory neurons in DRGs from WT animals (□) but not from GIP KO animals. GIP was found to enhance the Cap induced substance P secretion. NS, not significant, $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Summary and Future Study

The results of these experiments provide an extra-vagal pathway in which the peripheral tissues of the body and the CNS can sense IF contents via sensory nerves that innervate the lumen of the lymphatic vasculature. This pathway expands the function and effector response of GLP-1 and GIP as model molecules in the MLF/IF whose concentration is diet dependent. The extra hormonal and extra pancreatic action of GLP-1 and GIP can now include their ability to induce by the secretion of SP from primary cultured DRG neurons. In addition, it can be speculated that other neuropeptides, beside SP, are released in such stimulation, as it is known from the literature that TRPV1 expressing sensory neurons also contain other neuropeptides. Through these findings, it could be suggested that GLP-1, GIP and potentially other bioactive molecules in the MLF/IF could have effects on the peripheral tissue and the CNS though these neuropeptides, whose activation and secretion is controlled by their surrounding MLF/IF.

The investigation of this chemosensory system of the interstitium has opened doors to questions that remain to be answered. First and foremost is the question of lacteal innervation by sensory nerves of the DRG. To add evidence obtained through antibody staining, retrograde and /or anterograde tracing could be used to determine that spinal nerves enter the lumen of the lacteals. This will allow us to follow the nerves from the lacteals to the DRG, or DRG to epithelium of the lacteal. Other future experiments may include the investigation to: 1) determine the expression and localization of receptors for nutrient responsive peptides on sensory nerves, such as CCK-AR and PYY, 2) determine if activation of these receptors induce the release of neuropeptides, such as SP, CGRP, somatostatin, vasoactive intestinal polypeptide, etc., and 3) determine response of systemic lymph and compare/contrast to mesenteric lymph. The results of these suggested future studies could provide us a clear pathway whereby a specific organ can directly communicate with the CNS through its local lymph/IF and regulate an essential physiological function.

*The sections Methods, Results, and Discussion in this dissertation were originally prepared for a manuscript to be submitted for publication with my adviser as the corresponding author

References

1. Davenport, H. W. (1978) *A digest of digestion*, 2d ed., Year Book Medical Publishers, Chicago
2. Shenkin, A. (2006) Micronutrients in health and disease. *Postgrad Med J* **82**, 559-567
3. Williams, C. (1995) Macronutrients and performance. *J Sports Sci* **13 Spec No**, S1-10
4. Wolever, T. M. (2017) Effect of macronutrients on the glycemic index. *Am J Clin Nutr* **106**, 704-705
5. Walsh, J. H., Dockray, G. J., and Mitty, R. D. (1994) Gut Peptides: Biochemistry and Physiology. *The Endocrinologist* **4**, 487
6. Layer, P., Holst, J. J., Grandt, D., and Goebell, H. (1995) Ileal release of glucagon-like peptide-1 (GLP-1). Association with inhibition of gastric acid secretion in humans. *Dig Dis Sci* **40**, 1074-1082
7. Nguyen, C. A., Akiba, Y., and Kaunitz, J. D. (2012) Recent advances in gut nutrient chemosensing. *Curr Med Chem* **19**, 28-34
8. Dumoulin, V., Moro, F., Barcelo, A., Dakka, T., and Cuber, J. C. (1998) Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum. *Endocrinology* **139**, 3780-3786
9. Aponte, G. W., Fink, A. S., Meyer, J. H., Tatemoto, K., and Taylor, I. L. (1985) Regional distribution and release of peptide YY with fatty acids of different chain length. *Am J Physiol* **249**, G745-750
10. Sternini, C., Anselmi, L., and Rozengurt, E. (2008) Enteroendocrine cells: a site of 'taste' in gastrointestinal chemosensing. *Curr Opin Endocrinol Diabetes Obes* **15**, 73-78
11. Spiller, R. C., Trotman, I. F., Higgins, B. E., Ghatei, M. A., Grimble, G. K., Lee, Y. C., Bloom, S. R., Misiewicz, J. J., and Silk, D. B. (1984) The ileal brake--inhibition of jejunal motility after ileal fat perfusion in man. *Gut* **25**, 365-374
12. Engelstoft, M. S., Egerod, K. L., Holst, B., and Schwartz, T. W. (2008) A gut feeling for obesity: 7TM sensors on enteroendocrine cells. *Cell Metab* **8**, 447-449
13. Cordier-Bussat, M., Bernard, C., Levenez, F., Klages, N., Laser-Ritz, B., Philippe, J., Chayvialle, J. A., and Cuber, J. C. (1998) Peptones stimulate both the secretion of the incretin hormone glucagon-like peptide 1 and the transcription of the proglucagon gene. *Diabetes* **47**, 1038-1045
14. Cox, J. E., Kelm, G. R., Meller, S. T., and Randich, A. (2004) Suppression of food intake by GI fatty acid infusions: roles of celiac vagal afferents and cholecystokinin. *Physiol Behav* **82**, 27-33
15. Chaudhri, O., Small, C., and Bloom, S. (2006) Gastrointestinal hormones regulating appetite. *Philos Trans R Soc Lond B Biol Sci* **361**, 1187-1209
16. Wellendorph, P., Johansen, L. D., and Brauner-Osborne, H. (2009) Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients. *Mol Pharmacol* **76**, 453-465
17. Pal, K., Melcher, K., and Xu, H. E. (2012) Structure and mechanism for recognition of peptide hormones by Class B G-protein-coupled receptors. *Acta Pharmacol Sin* **33**, 300-311
18. Martin, J. F., van den Berg, M. A., Ver Loren van Themaat, E., and Liras, P. (2019) Sensing and transduction of nutritional and chemical signals in filamentous fungi: Impact on cell development and secondary metabolites biosynthesis. *Biotechnol Adv*

19. Gutkind, J. S. (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* **273**, 1839-1842
20. Ansari, K., Martin, S., Farkasovsky, M., Ehbrecht, I. M., and Kuntzel, H. (1999) Phospholipase C binds to the receptor-like GPR1 protein and controls pseudohyphal differentiation in Saccharomyces cerevisiae. *J Biol Chem* **274**, 30052-30058
21. Krantz, M., Becit, E., and Hohmann, S. (2006) Comparative genomics of the HOG-signalling system in fungi. *Curr Genet* **49**, 137-151
22. Rispail, N., Soanes, D. M., Ant, C., Czajkowski, R., Grunler, A., Huguet, R., Perez-Nadales, E., Poli, A., Sartorel, E., Valiante, V., Yang, M., Beffa, R., Brakhage, A. A., Gow, N. A., Kahmann, R., Lebrun, M. H., Lenasi, H., Perez-Martin, J., Talbot, N. J., Wendland, J., and Di Pietro, A. (2009) Comparative genomics of MAP kinase and calcium-calcineurin signalling components in plant and human pathogenic fungi. *Fungal Genet Biol* **46**, 287-298
23. Ma, D., and Li, R. (2013) Current understanding of HOG-MAPK pathway in Aspergillus fumigatus. *Mycopathologia* **175**, 13-23
24. Devi, L. A. (2005) *The G protein-coupled receptors handbook*, Humana Press, Totowa, N.J.
25. Sun, Q., and Zhao, Z. (2017) Peptide Hormones as Tumor Markers in Clinical Practice. *Enzymes* **42**, 65-79
26. Hutchinson, J. A., Burholt, S., Hamley, I. W., Lundback, A. K., Uddin, S., Gomes Dos Santos, A., Reza, M., Seitsonen, J., and Ruokolainen, J. (2018) The Effect of Lipidation on the Self-Assembly of the Gut-Derived Peptide Hormone PYY3-36. *Bioconjug Chem* **29**, 2296-2308
27. Dockray, G. J. (2014) Gastrointestinal hormones and the dialogue between gut and brain. *J Physiol* **592**, 2927-2941
28. Lach, G., Schellekens, H., Dinan, T. G., and Cryan, J. F. (2018) Anxiety, Depression, and the Microbiome: A Role for Gut Peptides. *Neurotherapeutics* **15**, 36-59
29. Kreymann, B., Williams, G., Ghatei, M. A., and Bloom, S. R. (1987) Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet* **2**, 1300-1304
30. Sandoval, D., and Sisley, S. R. (2015) Brain GLP-1 and insulin sensitivity. *Mol Cell Endocrinol* **418 Pt 1**, 27-32
31. Drucker, D. J., Habener, J. F., and Holst, J. J. (2017) Discovery, characterization, and clinical development of the glucagon-like peptides. *J Clin Invest* **127**, 4217-4227
32. Inagaki, N., Seino, Y., Takeda, J., Yano, H., Yamada, Y., Bell, G. I., Eddy, R. L., Fukushima, Y., Byers, M. G., Shows, T. B., and et al. (1989) Gastric inhibitory polypeptide: structure and chromosomal localization of the human gene. *Mol Endocrinol* **3**, 1014-1021
33. Brown, J. C. (1982) Gastric inhibitory polypeptide. *Monogr Endocrinol* **24**, III-XI, 1-88
34. Takeda, J., Seino, Y., Tanaka, K., Fukumoto, H., Kayano, T., Takahashi, H., Mitani, T., Kurono, M., Suzuki, T., Tobe, T., and et al. (1987) Sequence of an intestinal cDNA encoding human gastric inhibitory polypeptide precursor. *Proc Natl Acad Sci U S A* **84**, 7005-7008
35. Brown, J. C., Mutt, V., and Pederson, R. A. (1970) Further purification of a polypeptide demonstrating enterogastrone activity. *J Physiol* **209**, 57-64

36. Dupre, J., Ross, S. A., Watson, D., and Brown, J. C. (1973) Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J Clin Endocrinol Metab* **37**, 826-828
37. Adrian, T. E., Bloom, S. R., Hermansen, K., and Iversen, J. (1978) Pancreatic polypeptide, glucagon and insulin secretion from the isolated perfused canine pancreas. *Diabetologia* **14**, 413-417
38. Taminato, T., Seino, Y., Goto, Y., Inoue, Y., and Kadowaki, S. (1977) Synthetic gastric inhibitory polypeptide. Stimulatory effect on insulin and glucagon secretion in the rat. *Diabetes* **26**, 480-484
39. Seino, Y., Fukushima, M., and Yabe, D. (2010) GIP and GLP-1, the two incretin hormones: Similarities and differences. *J Diabetes Investig* **1**, 8-23
40. Seino, Y., and Yabe, D. (2013) Glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1: Incretin actions beyond the pancreas. *J Diabetes Investig* **4**, 108-130
41. Breit, S., Kupferberg, A., Rogler, G., and Hasler, G. (2018) Vagus Nerve as Modulator of the Brain-Gut Axis in Psychiatric and Inflammatory Disorders. *Front Psychiatry* **9**, 44
42. Ohlsson, L., Kohan, A. B., Tso, P., and Ahren, B. (2014) GLP-1 released to the mesenteric lymph duct in mice: effects of glucose and fat. *Regul Pept* **189**, 40-45
43. Wiig, H., and Swartz, M. A. (2012) Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. *Physiol Rev* **92**, 1005-1060
44. Bican, O., Minagar, A., and Pruitt, A. A. (2013) The spinal cord: a review of functional neuroanatomy. *Neurol Clin* **31**, 1-18
45. Leijnse, J. N., and D'Herde, K. (2016) Revisiting the segmental organization of the human spinal cord. *J Anat* **229**, 384-393
46. Li, Q., and Peng, J. (2014) Sensory nerves and pancreatitis. *Gland Surg* **3**, 284-292
47. Greco, R., Tassorelli, C., Sandrini, G., Di Bella, P., Buscone, S., and Nappi, G. (2008) Role of calcitonin gene-related peptide and substance P in different models of pain. *Cephalalgia* **28**, 114-126
48. Uddman, R., Edvinsson, L., Ekblad, E., Hakanson, R., and Sundler, F. (1986) Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. *Regul Pept* **15**, 1-23
49. Brain, S. D. (2004) Calcitonin gene-related peptide (CGRP) antagonists: blockers of neuronal transmission in migraine. *Br J Pharmacol* **142**, 1053-1054
50. Lembeck, F., and Holzer, P. (1979) Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. *Naunyn Schmiedebergs Arch Pharmacol* **310**, 175-183
51. Ho, K. W., Ward, N. J., and Calkins, D. J. (2012) TRPV1: a stress response protein in the central nervous system. *Am J Neurodegener Dis* **1**, 1-14
52. Chung, M. K., Guler, A. D., and Caterina, M. J. (2008) TRPV1 shows dynamic ionic selectivity during agonist stimulation. *Nat Neurosci* **11**, 555-564
53. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816-824
54. Negri, L., Lattanzi, R., Giannini, E., Colucci, M., Margheriti, F., Melchiorri, P., Vellani, V., Tian, H., De Felice, M., and Porreca, F. (2006) Impaired nociception and inflammatory pain sensation in mice lacking the prokineticin receptor PKR1: focus on

interaction between PKR1 and the capsaicin receptor TRPV1 in pain behavior. *J Neurosci* **26**, 6716-6727
55. Masuoka, T., Kudo, M., Yamashita, Y., Yoshida, J., Imaizumi, N., Muramatsu, I., Nishio, M., and Ishibashi, T. (2017) TRPA1 Channels Modify TRPV1-Mediated Current Responses in Dorsal Root Ganglion Neurons. *Front Physiol* **8**, 272
56. Tang, H. B., Inoue, A., Oshita, K., and Nakata, Y. (2004) Sensitization of vanilloid receptor 1 induced by bradykinin via the activation of second messenger signaling cascades in rat primary afferent neurons. *Eur J Pharmacol* **498**, 37-43
57. Szteyn, K., Rowan, M. P., Gomez, R., Du, J., Carlton, S. M., and Jeske, N. A. (2015) A-kinase anchoring protein 79/150 coordinates metabotropic glutamate receptor sensitization of peripheral sensory neurons. *Pain* **156**, 2364-2372
58. Masuoka, T., Nakamura, T., Kudo, M., Yoshida, J., Takaoka, Y., Kato, N., Ishibashi, T., Imaizumi, N., and Nishio, M. (2015) Biphasic modulation by mGlu5 receptors of TRPV1-mediated intracellular calcium elevation in sensory neurons contributes to heat sensitivity. *Br J Pharmacol* **172**, 1020-1033
59. Stanchev, D., Blosa, M., Milius, D., Gerevich, Z., Rubini, P., Schmalzing, G., Eschrich, K., Schaefer, M., Wirkner, K., and Illes, P. (2009) Cross-inhibition between native and recombinant TRPV1 and P2X(3) receptors. *Pain* **143**, 26-36
60. Logashina, Y. A., Korolkova, Y. V., Kozlov, S. A., and Andreev, Y. A. (2019) TRPA1 Channel as a Regulator of Neurogenic Inflammation and Pain: Structure, Function, Role in Pathophysiology, and Therapeutic Potential of Ligands. *Biochemistry (Mosc)* **84**, 101-118
61. Wang, D. H. (2005) The vanilloid receptor and hypertension. *Acta Pharmacol Sin* **26**, 286-294
62. Poole, D. P., Lee, M., Tso, P., Bunnett, N. W., Yo, S. J., Lieu, T., Shiu, A., Wang, J. C., Nomura, D. K., and Aponte, G. W. (2014) Feeding-dependent activation of enteric cells and sensory neurons by lymphatic fluid: evidence for a neurolymphocrine system. *Am J Physiol Gastrointest Liver Physiol* **306**, G686-698
63. Choi, S., Lee, M., Shiu, A. L., Yo, S. J., Hallden, G., and Aponte, G. W. (2007) GPR93 activation by protein hydrolysate induces CCK transcription and secretion in STC-1 cells. *Am J Physiol Gastrointest Liver Physiol* **292**, G1366-1375
64. Choi, S., Lee, M., Shiu, A. L., Yo, S. J., and Aponte, G. W. (2007) Identification of a protein hydrolysate responsive G protein-coupled receptor in enterocytes. *Am J Physiol Gastrointest Liver Physiol* **292**, G98-G112
65. Lee, M., Choi, S., Hallden, G., Yo, S. J., Schichnes, D., and Aponte, G. W. (2009) P2Y5 is a G(alpha)i, G(alpha)12/13 G protein-coupled receptor activated by lysophosphatidic acid that reduces intestinal cell adhesion. *Am J Physiol Gastrointest Liver Physiol* **297**, G641-654
66. Oh, D. Y., Yoon, J. M., Moon, M. J., Hwang, J. I., Choe, H., Lee, J. Y., Kim, J. I., Kim, S., Rhim, H., O'Dell, D. K., Walker, J. M., Na, H. S., Lee, M. G., Kwon, H. B., Kim, K., and Seong, J. Y. (2008) Identification of farnesyl pyrophosphate and N-arachidonylglycine as endogenous ligands for GPR92. *J Biol Chem* **283**, 21054-21064
67. Lin, M. E., Rivera, R. R., and Chun, J. (2012) Targeted deletion of LPA5 identifies novel roles for lysophosphatidic acid signaling in development of neuropathic pain. *J Biol Chem* **287**, 17608-17617

68. Preitner, F., Ibberson, M., Franklin, I., Binnert, C., Pende, M., Gjinovci, A., Hansotia, T., Drucker, D. J., Wollheim, C., Burcelin, R., and Thorens, B. (2004) Gluco-incretins control insulin secretion at multiple levels as revealed in mice lacking GLP-1 and GIP receptors. *J Clin Invest* **113**, 635-645
69. Hinman, A., Chuang, H. H., Bautista, D. M., and Julius, D. (2006) TRP channel activation by reversible covalent modification. *Proc Natl Acad Sci U S A* **103**, 19564-19568
70. Lopez-Requena, A., Boonen, B., Van Gerven, L., Hellings, P. W., Alpizar, Y. A., and Talavera, K. (2017) Roles of Neuronal TRP Channels in Neuroimmune Interactions. in *Neurobiology of TRP Channels* (nd, and Emir, T. L. R. eds.), Boca Raton (FL). pp 277-294
71. Jensen, E. P., Poulsen, S. S., Kissow, H., Holstein-Rathlou, N. H., Deacon, C. F., Jensen, B. L., Holst, J. J., and Sorensen, C. M. (2015) Activation of GLP-1 receptors on vascular smooth muscle cells reduces the autoregulatory response in afferent arterioles and increases renal blood flow. *Am J Physiol Renal Physiol* **308**, F867-877
72. Cuello, A. C., Galfre, G., and Milstein, C. (1979) Detection of substance P in the central nervous system by a monoclonal antibody. *Proc Natl Acad Sci U S A* **76**, 3532-3536
73. Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., and Julius, D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21**, 531-543
74. Farr, A. G., Berry, M. L., Kim, A., Nelson, A. J., Welch, M. P., and Aruffo, A. (1992) Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. *The Journal of experimental medicine* **176**, 1477-1482
75. Cottrell, G. S., Roosterman, D., Marvizon, J. C., Song, B., Wick, E., Pikios, S., Wong, H., Berthelier, C., Tang, Y., Sternini, C., Bunnett, N. W., and Grady, E. F. (2005) Localization of calcitonin receptor-like receptor and receptor activity modifying protein 1 in enteric neurons, dorsal root ganglia, and the spinal cord of the rat. *J Comp Neurol* **490**, 239-255
76. Aponte, G. W., Keddie, A., Hallden, G., Hess, R., and Link, P. (1991) Polarized intestinal hybrid cell lines derived from primary culture: establishment and characterization. *Proc Natl Acad Sci U S A* **88**, 5282-5286
77. McKemy, D. D., Neuhausser, W. M., and Julius, D. (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**, 52-58
78. Ulvmar, M. H., and Makinen, T. (2016) Heterogeneity in the lymphatic vascular system and its origin. *Cardiovasc Res* **111**, 310-321
79. Genton, L., and Kudsk, K. A. (2003) Interactions between the enteric nervous system and the immune system: role of neuropeptides and nutrition. *American journal of surgery* **186**, 253-258
80. Artis, D., and Spits, H. (2015) The biology of innate lymphoid cells. *Nature* **517**, 293-301
81. Gazzieri, D., Trevisani, M., Springer, J., Harrison, S., Cottrell, G. S., Andre, E., Nicoletti, P., Massi, D., Zecchi, S., Nosi, D., Santucci, M., Gerard, N. P., Lucattelli, M., Lungarella, G., Fischer, A., Grady, E. F., Bunnett, N. W., and Geppetti, P. (2007) Substance P released by TRPV1-expressing neurons produces reactive oxygen species that mediate ethanol-induced gastric injury. *Free Radic Biol Med* **43**, 581-589

82. Schicho, R., Florian, W., Liebmann, I., Holzer, P., and Lippe, I. T. (2004) Increased expression of TRPV1 receptor in dorsal root ganglia by acid insult of the rat gastric mucosa. *Eur J Neurosci* **19**, 1811-1818
83. Possenti, L., Casagrande, G., Di Gregorio, S., Zunino, P., and Costantino, M. L. (2019) Numerical simulations of the microvascular fluid balance with a non-linear model of the lymphatic system. *Microvasc Res* **122**, 101-110
84. Breslin, J. W., Yang, Y., Scallan, J. P., Sweat, R. S., Adderley, S. P., and Murfee, W. L. (2018) Lymphatic Vessel Network Structure and Physiology. *Compr Physiol* **9**, 207-299
85. Ichikawa, S., Kasahara, D., Iwanaga, T., Uchino, S., and Fujita, T. (1991) Peptidergic nerve terminals associated with the central lacteal lymphatics in the ileal villi of dogs. *Arch Histol Cytol* **54**, 311-320
86. Hukkanen, M., Konttinen, Y. T., Terenghi, G., and Polak, J. M. (1992) Peptide-containing innervation of rat femoral lymphatic vessels. *Microvasc Res* **43**, 7-19
87. Anand, U., Yiangou, Y., Akbar, A., Quick, T., MacQuillan, A., Fox, M., Sinisi, M., Korchev, Y. E., Jones, B., Bloom, S. R., and Anand, P. (2018) Glucagon-like peptide 1 receptor (GLP-1R) expression by nerve fibres in inflammatory bowel disease and functional effects in cultured neurons. *PLoS One* **13**, e0198024
88. Doyle, M. E., and Egan, J. M. (2007) Mechanisms of action of glucagon-like peptide 1 in the pancreas. *Pharmacol Ther* **113**, 546-593
89. Montrose-Rafizadeh, C., Avdonin, P., Garant, M. J., Rodgers, B. D., Kole, S., Yang, H., Levine, M. A., Schwindinger, W., and Bernier, M. (1999) Pancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells. *Endocrinology* **140**, 1132-1140
90. Quoyer, J., Longuet, C., Broca, C., Linck, N., Costes, S., Varin, E., Bockaert, J., Bertrand, G., and Dalle, S. (2010) GLP-1 mediates antiapoptotic effect by phosphorylating Bad through a beta-arrestin 1-mediated ERK1/2 activation in pancreatic beta-cells. *J Biol Chem* **285**, 1989-2002
91. Zhang, Q. H., Hao, J. W., Li, G. L., Ji, X. J., Yao, X. D., Dong, N., and Yao, Y. M. (2017) Proinflammatory switch from Galphas to Galphai signaling by Glucagon-like peptide-1 receptor in murine splenic monocyte following burn injury. *Inflamm Res*
92. Alexander, S. P., Mathie, A., and Peters, J. A. (2011) Guide to Receptors and Channels (GRAC), 5th edition. *Br J Pharmacol* **164 Suppl 1**, S1-324
93. Patel, T. D., Jackman, A., Rice, F. L., Kucera, J., and Snider, W. D. (2000) Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. *Neuron* **25**, 345-357
94. Li, C. L., Li, K. C., Wu, D., Chen, Y., Luo, H., Zhao, J. R., Wang, S. S., Sun, M. M., Lu, Y. J., Zhong, Y. Q., Hu, X. Y., Hou, R., Zhou, B. B., Bao, L., Xiao, H. S., and Zhang, X. (2016) Somatosensory neuron types identified by high-coverage single-cell RNA-sequencing and functional heterogeneity. *Cell Res* **26**, 83-102
95. Nascimento, A. I., Mar, F. M., and Sousa, M. M. (2018) The intriguing nature of dorsal root ganglion neurons: Linking structure with polarity and function. *Prog Neurobiol*
96. Julius, D. (2013) TRP channels and pain. *Annual review of cell and developmental biology* **29**, 355-384
97. Xu, Y., Jia, J., Xie, C., Wu, Y., and Tu, W. (2018) Transient Receptor Potential Ankyrin 1 and Substance P Mediate the Development of Gastric Mucosal Lesions in a Water Immersion Restraint Stress Rat Model. *Digestion* **97**, 228-239

98. Munoz, M., and Covenas, R. (2014) Involvement of substance P and the NK-1 receptor in human pathology. *Amino acids* **46**, 1727-1750
99. Selley, E., Kun, S., Szijarto, I. A., Kertesz, M., Wittmann, I., and Molnar, G. A. (2016) Vasodilator Effect of Glucagon: Receptorial Crosstalk Among Glucagon, GLP-1, and Receptor for Glucagon and GLP-1. *Horm Metab Res* **48**, 476-483
100. Asmar, A., Asmar, M., Simonsen, L., Madsbad, S., Holst, J. J., Hartmann, B., Sorensen, C. M., and Bulow, J. (2017) Glucagon-like peptide-1 elicits vasodilation in adipose tissue and skeletal muscle in healthy men. *Physiol Rep* **5**
101. Kant, V., Kumar, D., Prasad, R., Gopal, A., Pathak, N. N., Kumar, P., and Tandan, S. K. (2017) Combined effect of substance P and curcumin on cutaneous wound healing in diabetic rats. *The Journal of surgical research* **212**, 130-145
102. Spielman, L. J., Gibson, D. L., and Klegeris, A. (2017) Incretin hormones regulate microglia oxidative stress, survival and expression of trophic factors. *Eur J Cell Biol* **96**, 240-253
103. Holst, J. J., Burcelin, R., and Nathanson, E. (2011) Neuroprotective properties of GLP-1: theoretical and practical applications. *Curr Med Res Opin* **27**, 547-558
104. Harkavyi, A., and Whitton, P. S. (2010) Glucagon-like peptide 1 receptor stimulation as a means of neuroprotection. *Br J Pharmacol* **159**, 495-501
105. Kastrup, J., Henriksen, J. H., Pedersen, J. H., Fahrenkrug, J., Schaffalitzky de Muckadell, O. B., Schwartz, T., Parving, H. H., and Christensen, N. J. (1986) Effect of ganglionic blockade on endogenous circulating pancreatic polypeptide, vasoactive intestinal polypeptide, substance P, neurotensin and noradrenaline in healthy controls and long-term insulin-dependent diabetic patients. *Clin Sci (Lond)* **71**, 411-419
106. McMahon, S. B., and Wood, J. N. (2006) Increasingly irritable and close to tears: TRPA1 in inflammatory pain. *Cell* **124**, 1123-1125
107. Salas, M. M., Hargreaves, K. M., and Akopian, A. N. (2009) TRPA1-mediated responses in trigeminal sensory neurons: interaction between TRPA1 and TRPV1. *Eur J Neurosci* **29**, 1568-1578
108. Wang, Y., Parlevliet, E. T., Geerling, J. J., van der Tuin, S. J., Zhang, H., Bieghs, V., Jawad, A. H., Shiri-Sverdlov, R., Bot, I., de Jager, S. C., Havekes, L. M., Romijn, J. A., Willems van Dijk, K., and Rensen, P. C. (2014) Exendin-4 decreases liver inflammation and atherosclerosis development simultaneously by reducing macrophage infiltration. *Br J Pharmacol* **171**, 723-734
109. Shirazi, R., Palsdottir, V., Collander, J., Anesten, F., Vogel, H., Langlet, F., Jaschke, A., Schurmann, A., Prevot, V., Shao, R., Jansson, J. O., and Skibicka, K. P. (2013) Glucagon-like peptide 1 receptor induced suppression of food intake, and body weight is mediated by central IL-1 and IL-6. *Proc Natl Acad Sci U S A* **110**, 16199-16204
110. Holzer, P. (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* **24**, 739-768
111. Keller, A. C., Knaub, L. A., Miller, M. W., Birdsey, N., Klemm, D. J., and Reusch, J. E. (2015) Saxagliptin restores vascular mitochondrial exercise response in the Goto-Kakizaki rat. *J Cardiovasc Pharmacol* **65**, 137-147
112. Goud, A., Zhong, J., Peters, M., Brook, R. D., and Rajagopalan, S. (2016) GLP-1 Agonists and Blood Pressure: A Review of the Evidence. *Curr Hypertens Rep* **18**, 16

113. Lazar, B. A., Jancso, G., Oszlacs, O., Nagy, I., and Santha, P. (2018) The Insulin Receptor Is Colocalized With the TRPV1 Nociceptive Ion Channel and Neuropeptides in Pancreatic Spinal and Vagal Primary Sensory Neurons. *Pancreas* **47**, 110-115
114. Hibberd, T. J., Kestell, G. R., Kyloh, M. A., Brookes, S. J., Wattchow, D. A., and Spencer, N. J. (2016) Identification of different functional types of spinal afferent neurons innervating the mouse large intestine using a novel CGRPalpha transgenic reporter mouse. *Am J Physiol Gastrointest Liver Physiol* **310**, G561-573
115. Yabe, D., and Seino, Y. (2011) Two incretin hormones GLP-1 and GIP: comparison of their actions in insulin secretion and beta cell preservation. *Progress in biophysics and molecular biology* **107**, 248-256
116. Maggi, C. A. (1993) *The pharmacological modulation of neurotransmitter release*, New York: Harcourt Brace & Company, New York